University of Alberta

Identification of a Protein in Plants with Homology to Mammalian Serum Albumins

by

Cameron G. Lait ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

Department of Renewable Resources

Edmonton, Alberta

Fall 1998

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Date approved: July 14, 1998

ABSTRACT

Using polyclonal antibodies raised against human serum albumin (HSA), a 70 kD microsomal protein with an isoelectric point of 6.5 was detected in spinach (*Spinacia oleracea* L.), peas (*Pisum sativum)*, Scots pine (*Pinus sylvestris* L.), white spruce (*Picea glauca*), and canola (*Brassica napus*). The protein was purified by selective ammonium sulfate precipitation and anion exchange high performance liquid chromatography (HPLC). The protein from spinach shared 100% identity with the first fifteen NH_2-terminal amino acids of mature HSA. The protein from peas shared 70% identity with the first ten NH_2-terminal amino acids of HSA. The 70 kD plant protein contains the same NH_2-terminal X-X-H amino acid region which was identified in HSA as being responsible for binding of copper, zinc, indole derivatives, and calcium. The plant protein reacted positively with carbohydrate specific thymol stain and subsequent GC-MS data suggest the presence of associated galacturonic acid and galactose residues. The plant protein sequence contains the same indole binding site previously identified in HSA. $^{45}Ca^{++}$ overlay and 5-[7-^{3}H]azidoindole-3-acetic acid binding assay suggest that the purified serum albumin-like spinach protein binds both calcium and indole derivatives. Southern blot hybridizations to total spinach genomic DNA digested with Eco RV were achieved using biotin labeled synthetic oligonucleotides representing the highly conserved NH_2-terminal amino acid coding region of the HSA gene.

The protein was localized to the peripheral microsomal fraction using the Triton X-114 phase extraction method and later to the ER-Golgi system of spinach using continuous sucrose gradients and membrane enzyme markers. Nitrocellulose tissue prints probed with anti HSA antibodies provide immunological evidence suggesting that the serum albumin-like protein is secreted to the apoplast of cortex cells in plants. Localization and binding properties indicate that the plant protein identified in this dissertation may have a very basic cellular role as a transport or storage protein involved with the movement of precursors required for cell wall synthesis. We conclude that the 70 kD plant protein may be structurally or functionally related to serum albumin or other animal globin proteins, and represents another example of a plant protein with striking amino acid sequence homology to mammalian proteins.

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Acknowledgments

I am deeply indebted to my supervisor Dr. Janusz Zwiazek for the support of my graduate research at the University of Alberta. I was provided with an excellent research environment and consistent financial support, under sometimes difficult circumstances, with which I was able to complete my degree program.

I am grateful for the helpful advice and enthusiasm expressed by Dr. Zwiazek and the other members of my graduate committee, Dr. Marek Michalak and Dr. Francis Yeh.
I thank Dr. Abul Ekramoddoullah for traveling to Edmonton so he could serve as the external examiner for my defense and Dr. Om Rajora for serving on my examining committee.

Sincere thanks go to Dr. Marek Michalak, Dr. Francis Yeh, and Dr. Bruce Dancik for the use of their laboratory facilities during my research program. I acknowledge Dr. Alan Jones, from the University of North Carolina , for his kind assistance in the performance of the radioactive indole binding assay.
I wish to express special thanks to the many graduate students, research associates, and technical staff of Dr. Zwiazek's and Dr. Michalak's laboratories for helpful technical advice. I gratefully acknowledge the assistance of Jody Busaan and Mary Aleksiuk for sharing refined protocols and techniques gained from their years of experience.

My final thanks go to my family and the many friends which have given continuous support and encouragement throughout my university education.

Chapter One

1. General Introduction

In 1953, the first complete amino acid sequence of a protein was determined by Frederick Sanger and his colleagues (Sanger and Thomson 1953). The sequence determination of the 51 amino acids in insulin represented a milestone in protein research and for his work Sanger was awarded the Nobel Prize. In the decades following the amino acid sequence determination of insulin, scientists have been able to determine the primary structure of many other proteins of ever increasing size by using the peptide sequencing techniques of Pehr Edman (Edman 1970) and Frederick Sanger (Sanger et al. 1977). Many methods of enzymatic cleavage have been developed which cleave peptides at specific positions and allow amino acid sequence determination of the smaller fragments with exposed NH_2-termini using Edman degradation (Matsudaira 1990).

For many years following the sequencing of insulin, the new advancements in sequencing technology have allowed many other protein sequences to be compared between organisms. A general consensus was that two dissimilar organisms, and in some cases two species of a single genus, could only have a limited degree of amino acid sequence homology for any particular protein. Amino acid sequence homologies or identities, between plants and animals, exceeding 50% are still considered by some researchers as "striking" and unusual (Hsu et al. 1996; Lazar et al. 1995). However, recently an increasing number of proteins and translated mRNA sequences have been identified which exhibit high degrees of sequence homology between different organisms and, more interestingly, between the plant and animal kingdoms. There are numerous

examples of overall plant-human amino acid, or translated DNA sequence, identities of over 50%:

- Dihydrolipoamide acetyltransferase E2 subunit of the pyruvate dehydrogenase complex in *Arabidopsis thaliana* shares 57% amino acid sequence identity with its mammalian counterpart (Guan et al. 1995).

- Stress up-regulates a glyoxylase-I from a higher plant with 58% amino acid identity to mammalian glyoxylase (Espartero et al. 1995).

- Plants contain a protein with 59% amino acid sequence identity to mammalian splicing factor SF2/ASF (Lazar et al. 1995).

- Amino acid sequence identity of 76% was observed between a Ran-related GTP-binding protein coded by a plant cDNA and a mammalian homologue (Saalbach and Cristov 1994).

- A plant protein homologue with 79% sequence identity to mammalian GTPase Rab2 was identified in *Arabidopsis* (Moore et al. 1997).

- A plant protein homologous to the human Nm23 gene product was purified and had a striking 89% amino acid sequence identity with the human gene product (Sommer and Song 1994).

- ADP-ribosylation factor 1 cDNA isolated from *Chlamydomonas* encoded a protein with 90% amino acid sequence identity to the animal protein homolog (Memon et al. 1995).

The amino acid identities in key functional regions of some proteins exceed the overall identities described above, and in most cases the overall homologies are higher when conservative amino acid substitutions are considered.

It has been suggested that conserved amino acid sequences are essential for the proper function of a specific protein in a variety of different organisms. Pea (*Pisum sativum* L.) and human (*Homo sapiens*) class III alcohol dehydrogenase have an amino acid residue identity of 69% yet only 3 of 23 residues differ at the substrate and coenzyme binding sites (Shafqat et al. 1996). Cytochrome c has regions of amino acid sequence within its primary structure which remain unchanged in several organisms in order to conserve function (Rafferty et al. 1996; Caffrey et al. 1991). Despite the obvious structural differences between plant and animal cells, such as the cell walls, vacuoles and chloroplasts, many structural and functional similarities between plant and animal cells exist. The presence of mitochondria, endomembrane systems, plasma membranes, enzymes, nuclei, ribosomes and DNA are common to both plant and animal cells. There are many similarities in the overall biochemistry and basic structure and functions of both plant and animal cells. Many biochemical reactions in plant and animal cells involve similar enzymes and other proteins of highly specific structure and function. It is possible that some of the most highly conserved amino acid sequences, observed between different organisms, occur in proteins with very basic, and in some cases vitally important, cellular functions. Some examples of proteins, with regions of highly conserved amino acid sequence, which serve very basic cellular functions in both plant and animal cells include:

- Translation initiation factor 3 (Johnson et al. 1997) which is partly responsible for initiation of translation.

- Cell cycle gene enhancer (Gelsthorpe et al. 1997), implicated in the biosynthesis of pyrimidine.

- GTPase Rab 2 (Moore et al. 1997) which controls vesicle traffic between the endoplasmic reticulum and the Golgi apparatus.

- Adhesion molecule, (Zhang et al. 1996) critical to the maintenance and development of multicellular organisms.

- ADP-ribosylation factor 1, (Memon et al. 1995) involved with vesicle trafficking and signal transduction.

- COP9 and COP11 complexes which are conserved eukaryotic developmental regulators found in plants with highly conserved human counterparts (Chamovitz et al. 1996).

- Calreticulin, (Hassan et al. 1995) the major calcium storage protein in the endoplasmic reticulum.

One of the first reports of a substance in plants which had functional activity in vertebrate cells and exhibited possible structural similarity to an animal protein was an insulin-like substance reported in *Lemna gibba* (Collier et al. 1987). This discovery was ironic because the first amino acid sequence elucidated was that of mammalian insulin (Sanger and Thomson 1953). Hemoglobin, an important blood protein involved in respiratory gas transport in blood, has been identified in nodulating legume plants (Peive and Zhiznevskaia 1966; Wittenberg et al. 1975). Plant hemoglobin was later found in non-leguminous nodulating plant species (Christensen et al. 1991) and has also been reported in the roots of non-leguminous non-nodulating species (Bogusz et al. 1988). Structural similarity at both the protein and gene levels suggests a common evolutionary origin between animal and plant hemoglobins (Bogusz et al. 1988). It is believed that plant hemoglobins have a role in respiratory metabolism of root cells in all plant species

(Bogusz et al. 1988). Many other reports of plant proteins with striking (over 50%) homologies to human and other mammalian proteins were published more recently. In some cases, the presence of proteins in plants which are homologous to human proteins are not easily explained in the context of a possible functional role. Examples of human proteins for which puzzling plant homologues, of unknown function, have been identified include a human glioma-pathogenesis related brain tumor protein (Murphy et al. 1995), 14-3-3 brain protein (Stankovic et al. 1995), and a myosin-like protein (Moepps et al. 1993).

Following the identification of an insulin-like substance in plants using antibodies against vertebrate insulin (Collier et al. 1987), antibodies raised against mammalian proteins have been used to identify several homologous protein sequences in plants. Microsomes of tobacco (*Nicotiana tabacum*) cells contained two oxidative burst related proteins which were recognized by antibodies raised against human small G protein Rac2 (Kieffer et al. 1997). Two pectin binding proteins in *Cucurbita pepo* L. appear to be immunologically related to human vitronectin (Penel and Greppin 1996). Antibodies against human neutrophil p47-phox and p67-phox were used in immunoblots of several plants and were found to cross-react with proteins of the same molecular weight (Dwyer et al. 1996). A human fibrillarin was detected in onion cells (*Allium cepa*) using human autoimmune serum (Cerdido and Medina 1995) and NopA64 was detected in onion cells (*Allium cepa*) using a polyclonal antisera raised against mammalian nucleolin (de Carcer et al. 1997). A glycosylated form of calreticulin was identified in peas (*Pisum sativum* L.) using antisera raised against vertebrate calreticulin (Hassan et al. 1995). New proteins are

continuing to be identified in plants through the use of antibodies prepared against mammalian antigens.

In this dissertation, a polyclonal anti-human serum albumin antibody and a polyclonal anti-calreticulin antibody, that cross-reacts with serum albumin, were used to identify a 70 kD glycoprotein sharing NH_2-terminal amino acid sequence identity with human serum albumin. The glycoprotein with an isoelectric point of 6.5 was identified in several different plants and was localized to the endoplasmic reticulum-Golgi system of spinach (*Spinacia oleracea* L.). This protein may share some of the binding properties and subsequent functions observed in its human homologue, and represents another example of a highly conserved amino acid sequence in a plant protein with striking NH_2-terminal homology to a protein found in mammals.

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Chapter Two

2. Literature Review

The following is a compilation of literature pertaining to topics covered throughout this dissertation.

2.1 Plant proteins with homology to mammalian proteins

The physical characteristics, functions, and cellular localization of plant and animal proteins are influenced to a great extent by primary structure, the sequence of amino acids that make up the peptide chain. Secondary structure, the folding of a peptide chain, is also dictated largely by the position of key amino acid residues within a peptide. Secondary structures such as alpha-helices and beta-sheets can be formed by certain repetitive sequences of amino acids in a protein. Tertiary structure, the three dimensional structure of the entire polypeptide, dictates biological activity. Hydrophobic amino acids may be arranged in a sequence within an integral membrane protein at a position which spans a lipid bilayer. Signal peptides and retention signals are excellent examples of specific sequences which are conserved in different proteins. These signals control the passage of proteins to specific sub-cellular compartments and may ensure retention of proteins in others. The carboxy-terminal amino acid sequence KDEL (HDEL in plants) is an example of a retention signal which ensures proteins are retained in the endoplasmic reticulum (Munro and Pelham 1987). Amino acid sequences are critically important to the proper function of many proteins. Enzyme active sites, ion binding sites, hormone binding sites, and antigen recognition sites usually require correct positioning of charged

amino acid residues within a peptide and may involve "pockets" which allow a substrate access to internal regions of a peptide's active site. When a specific sequence of amino acids remains similar or identical in different proteins or in proteins found in two different organisms, it is said to be conserved. Amino acid sequence homology refers to the overall similarity of the amino acid sequence, tertiary structure, or function of a protein in two different organisms. For many years following the development of sequencing technologies, researchers were able to compare many different protein sequences. Homologies or identity between proteins which have similar or identical functions in different organisms were thought to be limited by the evolutionary similarity of those organisms. The same holds true for proteins found in both plants and animals. Alternate theories about similarities in amino acid sequence between disparate organisms suggest that large domains of conserved sequence originated in both plant and animal cells before the two kingdoms diverged and remained relatively unchanged over the course of evolution (Lopez et al. 1994; Ferl et al. 1994). Until the mid 1990's there were few reports of plant proteins with strikingly similar mammalian homologues. Homologies or identities, at the amino acid sequence level, of greater than 50% between plant and animal proteins were considered "striking" and unusual. However, recent literature suggests that there may be more examples of striking homologies between plant and animal proteins than once thought.

2.1.1 Conserved amino acid sequences in homologous proteins

Many examples of plant proteins with significant homologies to those found in humans or other mammals have been reported. However, the functions of many of these homologues in plants are not understood.

Antibodies are useful tools for recognition of specific amino acid sequences within a protein, and have lead to the discovery of plant-human protein homologues. In 1987 an insulin-like material was purified from spinach and *Lemna gibba* G3 (Collier et al. 1987). This plant protein had characteristics and activity that were very similar to vertebrate insulins but it was different chromatographically (Collier et al. 1987). Antibodies reacted with the insulin-like material, but its amino acid sequence was never compared to vertebrate insulin. Plant and animal cells produce similar oxidative burst responses to pathogen infection and one might expect to see similar oxidative burst proteins produced by cells from both kingdoms. Antibodies raised against the human neutrophil oxidative burst proteins p47-phox, p67-phox, and human small G protein Rac2 detected proteins in several plant extracts (Dwyer et al. 1996; Kieffer et al. 1997). Antibodies which were present in human autoimmune serum were described which detected a 37 kD nucleolar protein in onion (*Allium cepa*) (Cerdido and Medina 1995) and an antisera raised against mammalian nucleolin recognized a 64 kD protein in onion (*Allium cepa*) (de Carcer et al. 1997). Antibody cross-reactivity spanning kingdoms does not appear to be restricted to proteins with analogous functions. Tobacco (*Nicotiana tabacum*) infected with the tobacco mosaic virus produced a set of proteins, four of which cross-reacted with antibodies raised against a human enzyme 2'-5' A synthetase (Sher et al. 1990), a protein with a function apparently unrelated to viral infection of plants.

The discovery of some mammalian protein homologues in plants is not always explained in the context of conserved function. A substance with leuteinizing hormone releasing activity was purified from leaves of *Avena sativa* (Fukushima 1976). The purpose of a protein found in the central nervous system of animals, which was also identified in plants, insects, and yeast (Chen et al. 1994), is equally difficult to explain because plants lack a central nervous system. However, it is easier to speculate about the function of some protein homologues, such as those involved with basic developmental regulation. COP9 complex in plants represents a developmental regulator in higher eukaryotes (Chamovitz et al. 1996). COP9 and COP11 have closely related human counterparts (Chamovitz et al. 1996).

Amino acid sequence data analysis is another way by which homologous proteins can be compared between plants and animals. This information can suggest possible structural and functional similarities to other known proteins. A glutathione S-transferase (GST) was purified, from broccoli (*Brassica oleracea*), which had significant NH_2-terminal sequence homology to rat and human GST (Lopez et al. 1994).

A correlation between amino acid sequence and a protein's function in two different organisms is not always clear. Sommer and Song (1994) purified a nucleoside diphosphate kinase from *Avena* and obtained a 23 amino acid sequence at the NH_2-terminus. Of the 23 amino acids sequenced, 87% were identical to human Nm23 protein, a possible tumor metastasis suppressor (Sommer and Song 1994). Other examples of homologous amino acid sequences reported in both animals and plants include:

- Calreticulin, a major calcium binding protein in vertebrates which was purified from spinach (*Spinacia oleracea* L.) and pea plants (*Pisum sativum* L.) (Menegazzi et al.

1993; Hassan et al. 1995) and shares 55% identity with animal calreticulins (Napier et al. 1995).

- The highly conserved heat shock protein (HSP 90) amino acid sequence which has been compared between animals, plants and fungi revealing a minimum identity of 40% over the sequences of 31 different organisms (Gupta 1995).

- A pea (*Pisum sativum* L.) formaldehyde-active class III alcohol dehydrogenase with very high amino acid identity to human alcohol dehydrogenases (Shafqat et al. 1996).

- Protein kinase c interacting protein 1 (PKCI-1) which has sequence identity in a broad range of organisms including mycoplasma, plants, and humans (Lima et al. 1996).

2.1.2 Conserved DNA sequences which code for homologous proteins

Techniques in molecular biology have allowed researchers to compare large numbers of genes in plants and animals very rapidly. Traditional cloning techniques using cDNA expression libraries and the powerful Polymerase Chain Reaction (PCR) method have been used to screen for specific genes and for random clones from many plants and animals in recent years. Sophisticated computer software has made the comparison of similar gene sequences and the translation of the degenerate genetic code very easy and fast. By aligning gene sequences or translated protein products using one of several extensive computer databases such as the Basic Local Alignment Search Tool (BLAST), available via the internet, researchers are starting to discover genes, and proteins coded by genes in both plants and animals, which have extremely high similarity. Regions of

amino acid sequence identity between plant and animal proteins, and homologies much higher than previously thought to exist, are now being reported.

Functioning genes for haemoglobin were found in several plant species using cDNA probes (Bogusz et al. 1988; Anderson et al. 1996). Structural similarities at the gene and protein level led researchers to believe that animal and plant haemoglobins originated from a common evolutionary ancestor and have conserved functions in both plant and animal respiratory metabolism (Bogusz et al. 1988). Nematodes have a haemoglobin gene which contains an intron previously thought to be present only in plants (Dixon et al. 1992).

Shorrosh and Dixon (1991) cloned a DNA sequence from *Medicago sativa* which codes for protein with similarity to a vertebrate multifunctional protein disulfide-isomerase. This protein has several stretches of up to 16 consecutive amino acids within its sequence which are identical to the human homologue.

The gene coding for the largest proteasome subunit in *Arabidopsis* has a highly homologous mammalian counterpart (Shirley and Goodman 1993). The deduced amino acid sequence of the plant proteasome subunit exhibits 47% amino acid sequence identity with the mammalian form of the protein over nearly its entire length (Shirley and Goodman 1993).

Temperature induced proteins in plants can lead to the expression of an array of genes which would otherwise be relatively inactive. These and several other genes in plants have been identified which code for human and animal pathogenesis related proteins. Two low temperature induced genes *in Brassica napus* L. are very similar to the human tumour *bbc1* (breast basic conserved) gene (Saez-Vasquez et al. 1993).

Several plant proteins have been described which have homology to pathogenesis and tumor related proteins found in humans. A possible tumor metastasis suppressor, a nucleoside diphosphate kinase, was identified in *Avena* (Sommer and Song 1994). A highly conserved defender against apoptotic cell death gene in humans has plant and nematode homologs (Apte et al. 1995). A human defender against death [dad-1] cDNA homolog was isolated from rice plants (*Oryza sativum*) and appears to be well conserved between animals and plants (Tanaka et al. 1997). Human glioma pathogenesis-related protein gene expressed in brain tumours is closely related to plant pathogenesis-related proteins (Murphy et al. 1995). The gene for an adhesion protein in *Drosophila* belongs to a highly conserved family present in plants, yeast, nematodes and humans and is related to a human oncoprotein (Zhang et al. 1996).

Viral and bacterial infection resulted in the production of plant proteins with similarity to human proteins including some which are part of the immune system. LeMA-1 was cloned from tomato (*Lycopersicum esculentum*) infected with the tobacco mosaic virus and has high homology to the human protein TBP-1 (Prombona et al. 1995). A macrophage-specific membrane protein Nramp which controls natural resistance to bacterial infection has homologs expressed in the roots of plants (Belouchi et al. 1995). Several cDNA clones for enzymes present in both plants and animals have been isolated. In addition to being induced by the tobacco mosaic virus, LeMA-1 appears to be related to mammalian magnesium dependent ATPases and was isolated from tomato (*Lycopersicum esculentum*) cDNA clones (Prombona et al. 1995).

Proteins which serve similar metabolic functions have been reported to share homology between plants and animals. Dihydrolipoamide acetyltransferase (E2) subunit

of pyruvate dehydrogenase complex was cloned from *Arabidopsis* and has several stretches of deduced amino acid sequence identity with human pyruvate dehydrogenase subunits (Guan et al. 1995). Degenerate oligonucleotide primers, for conserved regions of a protein-L-isoaspartate O-methyltransferase in bacteria, wheat (*Triticum aestivum*), and human, were used to amplify a homologous enzyme in *Arabidopsis* (Mudgett and Clarke 1996). Two cDNA clones for casein kinase II catalytic subunit have been isolated from *Arabidopsis* which have a deduced amino acid homology of 72% with human casein kinase II (Mizoguchi et al. 1993). Cathepsin B-like protease cDNA with homology to human cathepsin B was isolated from *Nicotiana rustica* (Lidgett et al. 1995). A cDNA encoding a Ran-related GTP-binding protein was isolated from *Vicia faba* which has 76% identity to human Ran (Saalbach and Christov 1994). Glyoxalase 1 (GLX1) cDNA from tomato (*Lycopersicum esculentum*) codes for a protein which shares 58.5% identity with the human enzyme (Espartero et al. 1995). The gene for homogentisate dioxygenase has plant and human homologues (Fernandez-Canon and Penalva 1995). Cyclophilins encoded by rice (*Oryza sativum*) genes have been shown having 72% identity with human cyclophilins (Buchholz et al. 1994). Calreticulin cDNA clones were found in barley (*Hordeum vulgare*) which have 55% identity with animal calreticulins (Chen et al. 1994). A plant serine-arginine-rich protein with similarity to mammalian splicing factor SF2/ASF was identified by isolating a cDNA from *Arabidopsis* (Lazar et al. 1995). Very high sequence identity was observed between human and *Chlamydomonas* ADP-ribosylation factor 1 (Memon et al. 1995). The 20kD protein ADP-ribosylation factor 1 (ARF1) has a deduced amino acid sequence with 90% identity to human ARF1 (Memon et al. 1995). Delta 1-pyrroline-5-carboxylate reductase from *Zalerion arboricola* was

reported to share moderate amino acid sequence identity with plants, bacteria, yeast, and humans (Kelly and Register 1996).

The role of steroid hormones are important for developmental processes in animals, however the role of plant steroid hormones is relatively unknown. An *Arabidopsis* gene encodes a protein which shares significant sequence identity with mammalian steroid 5 alpha-reductases (Li et al. 1996).

Some genes were isolated from plants which code for proteins found only in specific human tissues. PCR was used to partially characterize a cDNA encoding a myosin-like protein in *Arabidopsis* (Moepps et al. 1993). A salt regulated plant gene was found that encodes a brain protein (Stankovic et al. 1995). Clones for the large subunit of eukaryotic translation initiation factor 3 which share significant identity have been isolated from tobacco (*Nicotiana tabacum*) and humans (Johnson et al. 1997).

A striking example of a plant gene coding for a protein sharing significant identity with its human homolog, is *Arabidopsis* RAB2 (Moore et al. 1997). Rab2 controls vesicle traffic between the endoplasmic reticulum and the Golgi apparatus of mammals. The *Arabidopsis* RAB2 deduced amino acid sequence shares 79% identity with human Rab2 (Moore et al. 1997).

2.2 Human serum albumin

Serum albumin is a multifunctional plasma protein which evolved by successive duplications of a gene fragment of primordial globin (Brown and Schockley 1982). The time of triplication of present-day albumin has been estimated at about 700 million years ago (Brown 1975). Serum albumin is a single copy gene (Hawkins and Dugaiczyk 1982)

and is part of a multigene family comprised of albumin, alpha-fetoprotein, alpha-albumin, and vitamin D-binding protein (Nishio et al. 1996).

Serum albumin is a 66 kD single chain protein of 585 amino acid residues, cross linked by 17 disulfide bridges into a series of nine loops, (Brown and Schockley 1982) and has a pI of 4.7-5.5 (Valmet 1969; Rosseneu-Motreff et al. 1970; Miller and Gemeiner 1993).

The physiochemical properties of both bovine and human serum albumins have been extensively studied. Serum albumin is thought to contain three major domains each divided into three subdomains with different binding properties (Brown and Schockley 1982). Principle binding sites include those for: long and short chain fatty acids (Spector and Fletcher 1978), steroids (Desgrez 1966), indole derivatives (Geisow and Beaven 1977) including indole acetic acid (Bertuzzi et al. 1997), uracil derivatives (Ochoa De Aspuru and Zaton 1993), bilirubin (Peters and Reed 1978), mercury (Truhaut 1966), copper (Shearer et al. 1967; Peters and Reed 1978), calcium, zinc, lead, magnesium, chloride and fluoride (McMenamy 1977). Serum albumin has been reported to have enzyme-like properties including esterase and peptidase activities (Taylor 1977; Kurono et al. 1984).

Many of the binding sites on the albumin protein reside near the NH_2-terminus as shown for calcium, indole and nickel binding (McMenamy 1977; Geisow and Beaven 1977). Nickel is thought to bind at two sites near residues 1-3 (Zhou et al. 1994) except in the extremely rare glycosylated form of serum albumin which doesn't bind nickel (Brennan et al. 1990). Serum albumin has been identified as the major binding protein in the transport of poly-beta-hydroxybutyrate (PHB) in human plasma (Reusch et al. 1992).

PHB is a an amphiphillic lipid found as a ubiquitous component of cellular membranes of bacteria, plants, and animals (Reusch et al. 1992). Serum albumins have been shown to bind strongly to tannins from various plant sources (Tsarevskii and Karal'nik 1975; Dawra et al. 1988).

Albumin is the most abundant protein in human plasma (Peters 1975), where it maintains blood osmotic pressure and acts as a multifunctional transport protein (Brown and Schockley 1982). Serum albumin is synthesized with a 24 amino acid leader peptide as "preproalbumin" in the polysomes associated with the endoplasmic reticulum of the liver. Preproalbumin is transported through the system of smooth endoplasmic reticulum into the Golgi bodies following the cleavage of 18 amino acids from its leader peptide. Mature albumin is then secreted following the removal of the remaining 6 amino acids of its leader peptide (Brennan et al. 1990).

Electrophoretic and immunological properties of serum albumins from several species have been studied. Electrophoretic mobilities indicating molecular weights ranging from 65.8 kD to 70.5 kD were observed for serum albumins of different species (Miller and Gemeiner 1993) and the rare glycosylated form of serum albumin (Brennan et al. 1990). Palmitic acid binding did not appear to alter the electrophoretic mobility of serum albumin (Miller and Gemeiner 1993) as was observed during isoelectric focusing of other proteins (Li and Ishibashi 1992). However, glycosylation caused serum albumin to appear 2.5 kD larger on SDS PAGE gels (Brennan et al. 1990).

Serum albumin is not easily visualized following SDS PAGE using silver as a staining method, therefore coomassie brilliant blue is preferred as a stain to prevent the observed "doughnut effect" (Miller and Gemeiner 1993). When antisera are raised

against serum albumin, most of the antigenic reactivity occurs at the terminal first third of the albumin molecule (Atassi et al. 1976). Further antigenic structural studies were performed on human serum albumin using monoclonal antibodies, produced against cyanogen bromide fragments of the protein. Some of the resulting monoclonal antibodies showed signs of cross-reactivity between different epitopes of the protein molecule (Doyen et al. 1985). The observation of intramolecular cross-reactivity of monoclonal antisera might be a result of similarities in repetitive antigenic domains despite differences in amino acid sequence.

2.3 Calreticulin

This literature review section is included because it was the search for the plant homologue of calreticulin which led to the discovery of the plant protein described in this dissertation.

Calreticulin was first described as a calcium (Ca^{2+}) binding protein in the sarcoplasmic reticulum of skeletal muscle (MacLennan and Wong 1971). Calreticulin has been described by many researchers under several names which include: high affinity Ca2+-binding protein (Ostwald and MacLennan 1974), calregulin (Waisman et al. 1985), calcium binding reticuloplasmin or CRP55 (Macer and Koch 1988), and calsequestrin-like protein (Volpe et al. 1988; Damiani et al. 1988). The molecular weight of calreticulin has almost as many reported values as it previously had names. The molecular weight estimate for calreticulin, based on the deduced amino acid sequence is 46 kD (Fliegel et al. 1989a). At neutral pH the protein's molecular weight was estimated at 55 kD by SDS PAGE (Ostwald and MacLennan 1974; Michalak et al. 1980). When calreticulin is

subjected to SDS PAGE as described by Laemmli (1970), the apparent molecular weight ranged between 60 kD and 63 kD (Waisman et al. 1985; Milner et al. 1991). Another characteristic of calreticulin subject to debate is its potential glycosylation. Despite the presence of a putative glycosylation site in calreticulin, there have only been reports of a few glycosylated forms of calreticulin (Waisman et al. 1985; Van et al. 1989; Hassan et al. 1995). The isoelectric point of calreticulin was measured at 4.65-4.67 (Waisman et al. 1985; McCauliffe et al. 1990a).

Calreticulin is thought to be a multifunctional protein because of its many physical characteristics. Calreticulin has been shown to bind calcium with both high and low affinities (Baksh and Michalak 1991) and zinc (Khanna et al. 1986; Khanna et al. 1987). Researchers have found calreticulin in sarcoplasmic reticulum and non-muscle endoplasmic reticulum (Opas et al. 1988; Fliegel et al. 1989b). Detergent and carbonate extraction have indicated that calreticulin is a peripheral membrane protein (Ostwald and MacLennan 1974; Michalak et al. 1980; Michalak et al. 1991).

Immunolocalization studies of calreticulin have identified calreticulin and what has been described as "calreticulin-like antigens" in the nuclei of some cells and throughout the endoplasmic reticulum system of cells (Opas et al. 1988; Opas et al. 1991). Cross-reactivity of some antibodies raised against calsequestrin with calreticulin has been observed (Volpe et al. 1988; Damiani et al. 1988; Krause et al. 1990; Treves et al. 1990). Calsequestrin and calreticulin share several biochemical characteristics including blue staining with the carbocyanine dye "Stains-all", pH sensitive electrophoretic mobilities and similar chromatographic properties (MacLennan et al. 1983; Fliegel et al. 1989b; Krause et al. 1990). It is possible that the observed cross-

reactivities are a result of difficulty in separating components with similar physical properties from the antigen mixture used in antibody production. This possibility is supported by the observation that calsequestrin and calreticulin share overall identity of only about 10% (Fliegel et al. 1989a; Scott et al. 1988; Milner et al. 1991).

Calreticulin cDNA clones have been identified in plants (Chen et al. 1994; Napier et al. 1995) and a calreticulin-like protein has been purified from spinach (*Spinacia oleracea* L.) leaves (Menegazzi et al. 1993). A glycosylated form of calreticulin was purified from *Pisum sativum* L. (Hassan et al. 1995).

2.4 Protein targeting and the ER-Golgi secretory system in plants

Several reviews have described, in detail, protein targeting and the ER-Golgi secretory system of eukaryotic cells (Akazawa and Hara-Nishimura 1985, Pfeffer and Rothman 1987, Chrispeels 1991, Pelham and Munro 1993, Staehelin and Moore 1995, Pelham 1996, Kermode 1996). With the exception of some structural features, such as the cell wall and vacuole, many similarities in structure and function exist between plant and animal cells. Organelles of the secretory system include the ER, Golgi complex, trans-Golgi network, endosomes, secretory vesicles, vacuoles, tonoplast, plasma membrane and various vesicles that make up transition structures between those listed above (Chrispeels 1991). The universality of protein targeting and secretion via the ER-Golgi system in a broad range of organisms from prokaryotes to eukaryotes and from the plant and animal kingdoms has been noted (Kermode 1996). Not surprisingly, key proteins involved in mammalian and yeast vesicle transport have close plant homologs (d'Enfert et al. 1992, Terryn et al 1993). Striking homology exists between two proteins involved with

yessicular traffic in evolutionarily divergent organisms. ARF1 from *Chlamydomonas* shares 90% amino acid sequence identity with human ARF1 (Memon et al. 1995) and Rab2 from *Arabidopsis* shares 79% amino acid sequence identity with human Rab2 (Moore et al. 1997). The exact mechanisms of protein targeting to specific compartments within plant cells and secretion of other proteins are not yet fully understood. However, some common primary structural features of proteins determine whether they will enter the secretory pathway, and where the proteins will eventually be delivered.

A signal peptide sequence is sufficient and necessary for a plant protein's entry into the secretory system of organelles (Chrispeels 1991). There are several types of amino acid signal sequences involved in protein targeting. These include: (1) signal sequences responsible for translocation across specific membranes into compartments such as the ER lumen, (2) stop transfer sequences which interrupt translocation initiated by signal sequences and can lead to regions of a protein which span a membrane bilayer, (3) retention signals which allow retention or recycling of a peptide within a specific compartment such as the KDEL/HDEL retention sequence characteristic of lumenal ER retained proteins, and (4) sorting signals which direct different final destinations of proteins which have been translocated along similar paths of the ER-Golgi network (Kermode 1996).

The Golgi apparatus in plants is a very important component of the secretory system. There are some notable differences between the ER-Golgi system of plants and animals. The Golgi stacks of plants are dispersed individually or in small clusters throughout the cytoplasm (Staehelin and Moore 1995). The ER of some plants can serve as a location for protein storage and may serve as a site for anchoring of the cytoskeleton

(Kermode 1996). The Golgi complex of plants differs from most other eukaryotic cells with respect to its role in the biosynthesis of polysaccharide components for the cell wall (Kermode 1996). In plants, the principle functions of the Golgi apparatus include the processing of complex polysaccharides for the cell wall, synthesis of glycolipids, transport of storage glycoproteins, and assembly or processing of oligosaccharide units for the cell wall (Staehelin and Moore 1995).

Very little is known about the molecular characteristics of the Golgi apparatus and the Trans Golgi Network of plants. Few transport proteins from within the Golgi have been purified to homogeneity and the genes which code for these proteins have not been well characterized (Staehelin and Moore 1995). The secretory system of plants is involved with the delivery of secretory proteins to vacuoles, the cell wall and the extracellular space (Chrispeels 1991). The types of proteins found in the secretory system of plants are quite diverse as are the conditions under which they are produced. Many examples of these secretory system proteins are summarized in reviews (Chrispeels 1991, Kermode 1996) and are briefly described below. Structural cell wall proteins such as: extensins over-expressed during pathogen invasion or wounding; hydroxyproline-rich glycoproteins [HRGPs] expressed during development, water stress, wounding or by abscisic acid; and auxin-regulated glycoprotein [AGPs] induced by wounding or by development, are reviewed by Showalter (1993). Some proteins including several enzymes are secreted by plants and include: amylases produced in response to hormonal signals, water stress, or calcium; chitinases produced during development or in response to fungal attack, and an auxin regulated glycoprotein secreted from suspension cultured carrot (*Daucus carota*) cells (Satoh and Fujii 1988; Van den Bulcke et al. 1989; Bol et al.

1990; Kermode 1990; Jones et al. 1993). Some proteins transported to vacuoles include: several examples of stress related proteins described in reviews by Chrispeels (1991) and Kermode (1996); vegetative and seed storage proteins (DeLisle and Crouch 1989; Mason and Mullet 1990; Staswick 1990; Wilen et al. 1990; Mason et al. 1992; Bethke and Jones 1994; Reinbothe et al. 1994; Jiang et al. 1995). Proteins such as developmentally regulated or stress induced TIP (Maurel et al. 1993) and slow vacuolar ion channel protein (Johnson et al. 1989) are targeted to the tonoplast membrane. Recently characterized secretory proteins transported to the plasma membrane include the defense-related pp34 (Jacinto et al. 1993) and developmentally or hormonally regulated plasma membrane ATPase (Michelet et al. 1994).

Glycosylation is a common post-translational or cotranslational feature of proteins found in the secretory system of plants and is very important for secreted and cell wall proteins of some plant species (De Vries et al. 1988). Two types of sugar linkage can occur during glycosylation, N-linked and O-linked. In N-linked glycosylation, the glycan chain is attached to the amide nitrogen of an asparagine residue. In O-linked glycosylation, the glycan chain is attached to the oxygen of either a serine or a threonine residue. The majority of enzymatic cell wall and membrane proteins in plants are N-glycosylated (Staehelin and Moore 1995). N-glycans are more complex than O-glycans, contain many branches, and may contain sugars in addition to the core mannose and glucose derivatives. In plants, the branched N-glycans may have additional fucose, xylose and galactose attached (Takahashi et al. 1986; Hayashi et al. 1990; Staehelin and Moore 1995). The two major classes of O-linked glycoproteins in plants are the hydroxyproline-rich glycoproteins [HRGPs] and the arabinogalactan proteins [AGPs] (Showalter 1993).

Most O-linked glycoproteins in plants are heavily glycosylated (50-98% of molecular mass) and serve structural and binding-recognition functions at the cell surface and within the cell wall (Staehelin and Moore 1995). O-linked glycans are composed mainly of arabinose and galactose but very few of these O-linked glycoproteins have been characterized (Bacic et al. 1987; Swords and Staehelin 1993).

Complex polysaccharides are primary products of the Golgi in growing plant cells (Staehelin and Moore 1995). Polygalacturonic acid/rhamnogalacturonan I (PGA/RGI) are the most abundant pectic polysaccharides in dicots. The backbone of RGI consists of alternating galacturonic acid and rhamnose components (Bacic et al. 1988). Galacturonic acid residues have also been identified in hemicelluloses. The biosynthesis of the complex polysaccharide components of cell walls has been localized to the Golgi compartments. As a result, several glycosyltransferases have been identified but few have been purified (Gibeaut and Carpita 1994). Several enzymes, which could possibly form multienzyme complexes, are involved with the addition of sugar residues to complex polysaccharides (Staehelin and Moore 1995). However, it is thought that independent enzymes are involved with the addition of fucose and galactose (Farkas and Maclachlan 1988). Few of the enzymes involved with the addition of monosaccharides to complex polysaccharides have been isolated and characterized.

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Chapter Three

3. Purification, and partial characterization of a 70 kD plant glycoprotein with NH_2-terminal amino acid sequence homology to human serum albumin.

3.1 Introduction

Recently several proteins, and genes coding for proteins, which share striking structural and amino acid sequence homology with human proteins have been identified in plants (Collier et al. 1987; Shorrosh and Dixon 1991; Mizoguchi et al. 1993; Saalbach and Christov 1994; Sommer and Song 1994; Apte et al. 1995; Guan et al. 1995; Lazar et al. 1995; Prombona et al. 1995). Some of the plant proteins, which are highly homologous to human proteins, share amino acid sequence identity in key structural or functional regions as evidenced by proteins with consecutive stretches of amino acids which are identical for: 83 residues (Memon et al. 1995), 27 residues (Prombona et al. 1995), 24 residues (Saalbach and Christov 1994), 17 residues (Mizoguchi et al. 1993; Sommer and Song 1994), 16 residues (Shorrosh and Dixon 1991) and several with multiple stretches showing identity of up to 9 consecutive residues (Apte et al. 1995; Guan et al. 1995; Lazar et al. 1995; Murphy et al. 1995). Many of the homologous proteins that plants and animals share are functionally related with very basic cellular roles such as respiration, transport, or enzymatic catalysis. Examples of such proteins include: ADP-ribosylation factor (Memon et al. 1995), proteasomes (Shirley and Goodman 1993), cathepsin (Lidgett et al. 1995), ATPases (Prombona et al. 1995), and calreticulin (Chen et al. 1994). However, there are examples of human proteins with

strikingly similar plant homologues whose functions are much less obvious. Included in this group of homologues are: glioma-pathogenesis related brain tumor protein (Murphy et al. 1995), 14-3-3 brain protein (Stankovic et al. 1995), a myosin-like protein (Moepps et al. 1993), and hemoglobin (Bogusz et al. 1988). Until now, there have been no reports of a protein related to serum albumin in plants.

Human serum albumin (HSA) is a 66kD multifunctional transport protein which is synthesized in the liver and is secreted into the bloodstream where it maintains the osmotic pressure of blood (Brown and Schockley 1982). Many of the primary binding characteristics of human serum albumin would be beneficial to plants. However, the role of these binding properties in humans is not well understood. Some of these binding properties include sites for long and short chain fatty acids (Spector and Fletcher 1978), indole derivatives and indole acetic acid (Geisow and Beaven 1977; Bertuzzi et al. 1997), calcium (McMenamy 1977), poly-beta-hydroxybutyrate (Reusch et al. 1992), and tannins (Dawra et al. 1988). Human serum albumin is not glycosylated except in extremely rare cases (Brennan et al. 1990).

In the present study, a 70 kD plant glycoprotein with amino acid sequence identical to human serum albumin over 15 residues at the NH_2-terminus and a pI of 6.5 was purified from spinach using HSA cross-reacting calreticulin polyclonal antibodies and commercial HSA polyclonal antibodies (Sigma Chemical Company) developed in goat. The NH_2-terminal amino acid sequence of the same protein purified from peas was also determined to be nearly identical to human serum albumin for the first 10 NH_2-terminal residues. Polyclonal HSA antisera recognized a single band of approximately 70 kD in HPLC purified proteins from spinach (*Spinacia oleracea* L.), canola (*Brassica*

napus L.), corn (*Zea mays* L.), black spruce (*Picea mariana* [(Mill.) B.S.P]), white spruce (*Picea glauca* [(Moench) Voss]), and Scots pine (*Pinus sylvestris* L.). This plant protein was partially characterized based on its observed binding properties.

3.2 Materials and Methods

3.2.1 Plant material

Protein used in HPLC purification was extracted from commercially grown spinach leaves (*Spinacia oleracea* L.), locally outdoor grown spinach leaves (*Spinacia oleracea* L. cv. Tyee) and spinach (*Spinacia oleracea* L. cv. Tyee) that was germinated on sterile growth media consisting of vermiculite and sand and grown in a greenhouse. Protein was also extracted from pea shoots (*Pisum sativum* L.) and canola (*Brassica napus* L.) which were germinated on sterile vermiculite and grown in growth chambers. Protein from Scots pine (*Pinus sylvestris* L.), black spruce (*Picea mariana* [(Mill.) B.S.P]), and white spruce (*Picea glauca* [(Moench) Voss]) was extracted from young needles of mature outdoor grown trees. Corn leaf (*Zea mays* L.) proteins were extracted from the leaves of mature outdoor grown plants.

3.2.2 Protein purification

Fresh spinach leaves (1 kg), pea shoots (500 g), canola leaves (500 g), coniferous (*Pinus sylvestris* L. or *Picea mariana* [(Mill.) B.S.P]) needles (200 g), or corn leaves (500 g) were homogenized in buffer (1 mL/g) containing 20 mM MOPS (pH 7.3) or 10 mM potassium phosphate (pH 7.1), 1 mM EGTA, 1 mM EDTA, 0.25 μM benzamidine, and 0.025 μM PMSF using a Waring blendor. The homogenate was filtered through 4 layers of cheesecloth and ammonium sulfate was added to 65% saturation. Following centrifugation at 14 300 x g, the supernatant was brought to 85% saturation by adding ammonium sulfate and the pH was adjusted to 4.5 using HCl. The solution was stirred for

2 hr at 4 ^{0}C and centrifuged at 14 300 x g for 45 min. The resulting pellet was dissolved in a minimal volume of deionized water and the pH adjusted to 7.3. Dialysis was performed overnight against deionized water. Proteins were precipitated and defatted using a chloroform/methanol/water (1/4/3, by volume) phase mixture as described by Pohl (1990). The phase mixture was vortexed and centrifuged briefly at 1 500 x g to separate the phases. The upper phase was discarded, leaving precipitate (protein) at the interface, and 3 volumes of methanol was added to the remaining chloroform phase. After centrifugation at 1 500 x g for 15 min. the protein pellet was recovered and dried under vacuum. Dried protein was dissolved in 20 mM MOPS buffer, pH 7.3 containing 0.5 mM DTT, 1 mM EGTA, and 1 mM EDTA.

Protein samples partially purified by selective ammonium sulfate precipitation (above) were separated by anion exchange HPLC using a 6 mL Resource™ Q anion exchange column (Pharmacia LKB Biotechnology Baie D'Urfe, PQ, Canada). Buffers used to elute proteins were: Buffer A 20 mM MOPS, pH 7.3, containing 0.5 mM DTT, 1 mM EGTA, and 1 mM EDTA; Buffer B 20 mM MOPS, pH 7.3, containing 0.5 mM DTT, 1 mM EGTA, 1 mM EDTA and 1 M NaCl. The following gradient was used to elute proteins: time 0-6 min, 0-1% buffer B; time 6-36 min, 1-50% buffer B, flow rate of 1 mL min^{-1}. Fractions (1 mL) were collected using the Bio-Rad 2110 fraction collector (Bio-Rad Laboratories, Mississauga, ON, Canada) and prepared for electrophoresis.

Protein quantity estimation was performed using the bicinchoninic acid (BCA) protein assay (Pierce Chemical).

3.2.3 Electrophoresis

One dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed as described by Laemmli (1970) with a 10% acrylamide resolving gel using the Bio-Rad Mini-Protean II apparatus. Two dimensional electrophoresis was performed as described by O'Farrell (1975) using the Bio-Rad Mini-Protean II 2-D system. All proteins were precipitated using a chloroform/methanol/water phase system as described for protein purification.

Proteins used for SDS PAGE were dissolved in 1-D PAGE sample buffer containing 60 mM Tris-HCl (pH 6.8), 4% SDS, 0.1 M DTT, 10% 2-mercaptoethanol, 20% glycerol and 0.01% bromophenol blue. All samples were loaded on gels using sterile, disposable gel loader pipet tips (Bio-Rad Laboratories). Proteins used for 2-D PAGE were dissolved in 2-D PAGE sample buffer containing 9.5 M urea, 2% (w/v) Triton X-100, 5% 2-mercaptoethanol, 1.6% 5/8 ampholyte, 0.4% 3/10 ampholyte. Isoelectric point (PI) of spots was determined using a micro-electrode to measure the pH of 2-D PAGE gel segments for each gel and were confirmed with pH measurements of gel segments from 2-D PAGE gels containing IEF standards (Sigma Chemical Company).

Following electrophoresis, some gels were electroblotted while others were stained with coomassie blue, thymol-sulfuric acid (Racusen 1979), Stains-all (Campbell et al. 1983), or silver stains.

3.2.4 Electroblotting, immunodetection and amino acid sequencing

Proteins were transferred from PAGE gels to nitrocellulose or Immobilon™ polyvinylidene difluoride (PVDF) membranes according to the electroblotting methods of Towbin et al. (1979) and Matsudaira (1987), respectively. Nitrocellulose membranes were blocked overnight in Tris-buffered saline containing 5% (w/v) non-fat milk powder.

Proteins transferred to nitrocellulose membranes were probed with primary polyclonal antibody (1:500) raised in goat against human serum albumin, monoclonal antibody (1:5000) raised in mouse against human serum albumin (Sigma Chemical Company), or polyclonal antibody (1:500) raised in goat against calreticulin. Secondary antibody (1:3000) conjugated to horseradish peroxidase was used to label immunoreactive bands for visualization using enhanced chemiluminescence (Amersham Life Sciences, Oakville, ON, Canada) or antibody conjugated to alkaline phosphatase (1:5000) for visualization using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) in 100 mM Tris buffer pH 9.5 containing 100 mM NaCl, 5 mM $MgCl_2$. Antibodies were diluted in Tris-buffered saline containing 1% (w/v) non-fat milk powder and 0.05% Tween 20 (TTBS).

NH_2-terminal amino acid sequencing of the 70 kD protein band was carried out on HPLC purified protein from spinach leaves and pea shoots. All protein samples were subjected to selective ammonium sulfate precipitation followed by purification using anion exchange HPLC and electroblotting to Immobilon PVDF membrane prior to sequencing. Automated amino acid sequence analyses were performed with an Applied Biosystems (Foster City, CA) Model 120A PLC, using Applied Biosystems protocols.

3.2.5 Stains-all staining and ^{45}calcium overlay

The cationic carbocyanine dye "Stains-all" was used to stain SDS PAGE gels as described by Campbell et al. (1983). $^{45}Ca^{2+}$ binding overlays were performed by the method of Maruyama et al. (1984) following transfer of proteins to nitrocellulose. Nitrocellulose membranes blotted with, HPLC purified and SDS PAGE separated, spinach proteins were washed for 3 hr with 3 changes of blocking buffer consisting of 60 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole-HCl, pH 6.8. Membranes were incubated in blocking buffer containing 2.02 μCi/mL ^{45}Ca for 10 min. Unbound ^{45}Ca was rinsed away with distilled water for 5 min. and nitrocellulose membranes were allowed to dry before exposure to X-ray film.

3.2.6 Auxin binding assay

Spinach protein was purified as described in section 3.2.2 (above) and photoaffinity labeling *in vitro* with 5 μM 5-[^{3}H]N_3IAA was performed as described by Brown and Jones (1994). Separate photoaffinity labeling reactions were performed on 10 μg aliquots of purified protein, at pH 5.5 and pH 7.0 in absence of exogenous unlabeled IAA (control), and at each pH in the presence of 10 μM, 50 μM, and 100 μM unlabeled IAA. All reaction mixtures were incubated for 3 hours prior to SDS PAGE. Gels were dried on filter paper and exposed to X-ray film for 90 days in an autoradiograph cassette.

3.2.7 Carbohydrate analysis, gas chromatography - mass spectrometry

SDS PAGE gels were stained for carbohydrate using thymol and sulfuric acid as described by Racusen (1979).

Monosaccharide composition analysis, following cleavage by methanolysis, was carried out by gas chromatography-mass spectrometry (GC-MS) according to Merkle and Poppe (1994). An HPLC purified protein sample containing about 50 μg of protein was separated by SDS-PAGE, the immunoreactive protein band was excised, and the gel slice was macerated in a minimal volume of methanol. Myo-inositol was added as an internal standard and the mixture was dried under a stream of nitrogen. To the dried sample, 500 μL of 1 M methanolic HCl was added and incubated for 16 hr at 80 ^{0}C. Methanol was evaporated under nitrogen and the sample was N-acetylated by adding 200 μL of methanol, 40 μL of pyridine and 40 μL of acetic anhydride. The tube was sealed with a teflon-lined cap and incubated overnight at room temperature. The solvents were evaporated under a stream of nitrogen and the sample was silylated by dissolving it in 200 μL of pyridine, 20 μL of trimethylchlorosilane and 20 μL of hexamethyldisilazane. Gas chromatography of silylated methylglycosides was performed by capillary GC using a Hewlett Packard 5890 gas chromatograph. The sample was separated on a 30 m long, 0.25 mm i.d. fused silica DB-5 capillary column, 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). Conditions during the run were as follows: oven temperature 190 ^{0}C, injector and detector temperature 250 ^{0}C, 50:1 split injection, carrier gas (He) linear flow rate of 25 cm s^{-1}. Carbohydrates separated by GC were identified

from their mass spectra using a VG 7070E mass spectrometer (Vacuum Generators, Manchester, UK), EI mode, 70 eV.

3.2.8 Chemicals and supplies

All chemicals were of the highest grade available and were purchased from Sigma Chemical Company. Immobilon PVDF membrane was obtained from Bio-Rad Laboratories and nitrocellulose membrane was obtained from Micron Separations Inc., (Westboro, MA, USA). Prestained molecular weight standards were obtained from Bio-Rad Laboratories and Sigma Chemical Company. Anti-calreticulin antibody was supplied by Dr. M. Michalak (Department of Biochemistry, University of Alberta, Edmonton, Canada).

3.3 Results

3.3.1 Cross-reactivity of calreticulin antisera

In addition to recognizing calreticulin in plants (Hassan et al. 1995), the polyclonal antibody raised in goat against purified animal calreticulin showed cross-reactivity with a larger 70 kD protein in peas and spinach. A number of microsomal membrane preparations isolated from different plant species were tested by immunoblotting with the anti-calreticulin antiserum. Figure 3-1A shows that anti-calreticulin antiserum recognised two protein bands of approximately 60 kD and 70 kD in plant protein extracts. When microsomal membrane extracts from spinach were probed with the anti-human serum albumin (HSA) polyclonal antiserum, the 70 kD band was clearly present with a smear below it (Fig. 3-1B). Amino acid sequencing results of the purified 70 kD pea protein which cross-reacted with the anti calreticulin antibody showed a high similarity to the human serum albumin sequence (Fig. 3-6). Results shown in Fig. 3-2 confirm suspected cross-reactivity of the calreticulin antibody with human serum albumin. While the calreticulin antibody recognized both calreticulin and human serum albumin (Fig. 3-2B), the anti human serum albumin antibody only recognized the protein against which it was raised (Fig. 3-2C) and the 70 kD protein purified from peas and spinach. Under the experimental conditions, neither the calreticulin antibody nor the human serum albumin antibody recognized bovine serum albumin (Fig. 3-2B and Fig. 3-2C). Monoclonal antibodies raised against human serum albumin did not recognize the purified plant protein (Fig. 3-2A).

3.3.2 Protein purification

A 70 kD serum albumin-like protein was purified from spinach leaves using selective ammonium sulfate precipitation followed by anion exchange HPLC. The HPLC elution profile is shown in Fig. 3-3A and the corresponding HSA antisera probed immunoblots of pooled pairs of fractions are shown in Fig. 3-3B. The serum albumin-like plant protein elutes at a sodium chloride concentration of 200-250 mM. Column flow through and all other HPLC fractions containing protein were blotted and probed with HSA antisera but showed no immunoreactive bands. When the HPLC purification procedure was repeated using the 35%-65% saturated ammonium sulfate precipitated fraction, no immunoreactive protein bands were observed in the elution profile when the anti human serum albumin antibody was used to screen the resulting HPLC fractions (results not shown).

3.3.3 Amino acid sequence

Fig. 3-6 shows the sequence obtained for the first fifteen amino acids at the NH_2-terminus of the HPLC purified serum albumin-like protein from spinach and the first ten amino acids of the serum albumin-like protein purified from peas. The spinach and pea protein sequences were aligned with human serum albumin showing a high degree of conservation in the region of the NH_2-terminus.

3.3.4 Two dimensional electrophoresis, electroblotting and isoelectric point determination

HPLC fractions containing the partially purified serum albumin-like spinach protein were pooled and subjected to two dimensional PAGE (Fig. 3-4A). A second two dimensional PAGE gel of HPLC purified serum albumin-like spinach protein was electroblotted to nitrocellulose and probed using polyclonal HSA antisera (Fig. 3-4B). The two dimensional western blot of the spinach proteins revealed the presence of a single spot with an approximate isoelectric point of 6.5, flanked by two less conspicuous spots with isoelectric points of approximately 6.4 and 6.6 respectively.

A two dimensional PAGE gel with commercially purified human serum albumin was also electroblotted and probed with polyclonal HSA antisera (Fig. 3-4C) to allow a comparison of its spotting pattern and focusing properties with those of the plant protein. Human serum albumin also showed 3 or 4 immunoreactive spots but appeared to have lower approximate isoelectric points.

3.3.5 Stains-all and calcium binding

The cationic carbocyanine dye "Stains-all" stained the HPLC purified 70 kD serum albumin-like plant protein blue, indicating possible calcium binding properties (Fig. 3-5B). Human serum albumin and bovine serum albumin standards stained negatively with "Stains-all" despite the reported presence of a calcium binding site (McMenamy 1977). ^{45}Calcium overlay of HPLC purified protein blotted to nitrocellulose

showed a positively reacting band corresponding to the position of the 70 kD serum albumin-like protein (Fig. 3-5D).

3.3.6 Auxin binding assay

The serum albumin-like protein bound the auxin analog 5-[^{3}H]N_3IAA at pH 7.0 (Fig. 3-8). This protein appeared to show a decrease in the amount of radiolabeled analog binding in the presence of increasing amounts of exogenous, unlabeled, IAA competitive inhibitor.

3.3.7 Carbohydrate analysis, gas chromatography - mass spectrometry

HPLC fractions containing plant serum albumin-like protein were analyzed by electrophoresis and stained positively with thymol-sulfuric acid stain, indicating the presence of carbohydrates (Fig. 3-5C). Human serum albumin and bovine serum albumin standards stained negatively with thymol-sulfuric acid. Plant protein bands which were both immunoreactive with HSA antisera and stained with thymol were cut from gels and used for GC-MS characterization. Data obtained from mass spectra of silylated methylglycosides (Appendix 1) indicated the presence of galactose and traces of galacturonic acid in association with the purified plant protein.

3.3.8 Immunoreactive proteins in different species

Polyclonal antibodies raised against human serum albumin reacted with a protein band of approximately 70 kD in several different plant species (Fig. 3-7). Dicotyledonous and coniferous plant species shown to contain the immunoreactive protein include peas, spinach, Scots pine, black spruce, white spruce, and canola. The immunoreactive protein band was also observed in a monocot (*Zea mays* L.) following purification using the same ammonium sulfate precipitation procedure and HPLC protocols described in this chapter for spinach. The immunoreactive protein was observed in all tissues studied for spinach, including: roots, shoots, and leaves (not shown).

3.4 Discussion

A 70 kD glycoprotein with striking NH_2-terminal amino acid sequence similarity to mammalian serum albumins has been identified in spinach leaves and pea shoots. The 70 kD serum albumin-like plant protein was observed using polyclonal antibodies raised against vertebrate calreticulin which exhibited cross-reactivity with human serum albumin (Fig. 3-2) and polyclonal antisera raised against human serum albumin.

The protein was partially purified (Fig. 3-3) and successfully sequenced on three separate occasions using spinach from different sources. Details about the difficulties encountered during the development of the purification strategy are included in Chapter 4. On all three occasions, the sequence obtained for the first fifteen amino acids of the spinach protein was identical to the NH_2-terminus of human serum albumin (Fig. 3-6). The serum albumin-like protein identified in peas had an NH_2-terminal amino acid sequence that was similar to the one obtained for spinach, as shown in Fig. 3-6.

Several researchers have recently identified proteins with conserved amino acid sequences, structures and functions in plant and human cells. This report of a serum albumin-like protein in spinach is not the first example of NH_2-terminal amino acid sequence identity shared between human and plant proteins. Human ADP-ribosylation factor 1 shares consecutive identical amino acid sequence with *Arabidopsis* and *Maize* from residue 15 to 97 (Memon et al. 1995). Other plant proteins which share conserved amino acid sequences with human proteins include: a dihydrolipoamide acetyltransferase [E2] subunit of the pyruvate dehydrogenase complex isolated from *Arabidopsis thaliana* (Lazar et al. 1995), a cathepsin B-like protease from *Nicotiana rustica* (Lidgett et al. 1995), the Defender Against Death 1 [DAD1] gene product in *O. sativa* (Apte et al. 1995;

Tanaka et al. 1997), a plant serine-arginine-rich protein with striking sequence and structural homology to the mammalian splicing factor SF2/ASF isolated from *Arabidopsis* (Lazar et al. 1995), a human glioma pathogenesis-related protein with similar functions in plants and humans (Murphy et al. 1995), a tomato (*Lycopersicum esculentum*) protein [LeMA-1] belonging to the SEC18-PAS1-CDC48-TBP-1 protein family of putative Mg^{2+} dependent ATPases (Prombona et al. 1995), Ran-related GTP-binding protein from *Vicia faba* (Saalbach and Christov 1994), calreticulin in barley (*Hordeum vulgare*) (Chen et al. 1994), a nucleoside diphosphate kinase in *Avena* (Sommer and Song 1994), the proteasome subunit from *Arabidopsis* (Shirley and Goodman 1993), casein kinase II catalytic subunits in *Arabidopsis* (Mizoguchi et al. 1993), and a putative plant endomembrane protein resembling vertebrate disulfide isomerase and a phosphatidylinositol-specific phospholipase C (Shorrosh and Dixon 1991).

Plant proteins and peptides which have similar functional activity in humans include: an insulin like material purified from spinach and *Lemna gibba* G3 (Collier et al. 1987), a leuteinizing hormone-releasing hormone-like substance from oak (*Quercus*) leaves (Fukushima et al. 1976), and a somatostatin-like material (Werner et al. 1985).

The degree to which the NH_2-terminal amino acid sequences are conserved in human serum albumin and the serum albumin-like protein from spinach might give some clue as to a possible function in plants. Several binding regions reside at the NH_2-terminus of human serum albumin including an indole binding site and several divalent cation binding sites (Geisow and Beaven 1977). Indole is a chemical structure which is present in the plant hormone indole acetic acid (IAA), and the binding of divalent cations

is important in many biochemical processes in both plants and humans. The binding of an indole derivative by the serum albumin-like plant protein was demonstrated (Fig. 3-8). Tian et al. (1995) demonstrated that an auxin binding receptor (ABP-1) has an auxin binding affinity optimum at pH 5.5 while the receptor is predominantly localized to the ER where the pH is closer to 7.0. In the present study, IAA binding by the serum albumin-like protein was demonstrated at pH 7.0 but was inhibited at increased concentration of IAA. Binding of the indole derivative 5-[^{3}H]N_3IAA at pH 7.0 suggests that the serum albumin-like plant protein might have binding activity in the lumen of the ER. Interestingly, Bertuzzi et al. (1997) demonstrated that human serum albumin binds the common phytohormone IAA and may have more than one indole binding site.

The cationic carbocyanine dye "Stains-all", stained the serum albumin-like plant protein blue, indicating the presence of amino acid residues which potentially bind calcium. A ^{45}calcium overlay was performed with spinach proteins prepared by several different methods. Piemonte et al. (1993) observed that while 1% Triton X-100 caused aggregate formation, enzyme activity of glutathione S-transferase increased when the detergent was present. Similarly, the plant protein appeared to bind calcium when Triton X-100 was present during purification (Fig. 3-5D).

Calcium binding is a characteristic of serum albumins (McMenamy 1977), however, the role of calcium binding to HSA is not understood. The role of calcium in plant growth, signal transduction, and development processes is complex, and calcium binding proteins play an important role in storage, transport, and accumulation of calcium ions which, in plants, are normally present in millimolar quantities (Hepler and Wayne 1985)

Binding of galactose and galacturonic acid, and polyphenolics by the spinach serum albumin-like protein, as indicated by GC-MS data (Appendix 1), may suggest a basic role for the protein in transport of sugars or other materials involved in the synthesis of cell walls.

The calcium and indole binding shown by the serum albumin-like plant protein suggests possible regulation of the protein's action by intracellular calcium ions and (or) by indole acetic acid. It is possible that IAA is transported to cell walls by the HSA-like protein where it participates in cell wall loosening and increased extensibility as described by Rayle and Cleland (1970). Alternatively, the plant protein may serve a more general role associated with the storage or transport of these and other substances.

Due to the extremely high sequence similarity, and the reactivity of the purified plant protein with polyclonal human serum albumin antisera, the possibility of contamination with exogenous sources of human serum albumin was considered. However, this was eliminated using a number of precautions. All equipment used in the purification of the plant proteins in these experiments has been dedicated for use with plant material only and mammalian tissues were never used in the laboratory. Human serum albumin that was used as standards for electrophoresis was never used on protein sequencing gels or in any apparatus that was used for the purification of proteins for sequencing. Additionally, despite identical NH_2-terminal amino acid sequences in both human serum albumin and the protein described in spinach, there are several fundamental differences in the properties of the two proteins. The spinach serum albumin-like protein is glycosylated and binds galacturonic acid, whereas human serum albumin is glycosylated only in extremely rare cases (Brennan et al. 1990).

The measured isoelectric point of human serum albumin ranges from 4.7 to 5.5, while that of the plant protein was measured at between 6.4 and 6.6 using 2-D PAGE. The observed 2-D PAGE spotting pattern of the spinach protein differs from human serum albumin. (Fig. 3-4). The molecular mass of the spinach protein is greater than that of HSA, appearing to migrate at 70 kD on a 10% SDS PAGE gel compared to 66 kD for HSA. The "Stains-all" staining of the spinach protein yields a blue band which differs from the observed staining of HSA (Fig. 3-5B).

Monoclonal antisera raised against human serum albumin did not recognize the plant protein (Fig. 3-2A), indicating possible differences between the antigenic structures of the plant protein and its human homologue.

Anion column flow-through and fractions containing other proteins derived from HPLC separations were probed with HSA antisera and showed no immunoreactive bands, thus serving as negative controls and discounting the likelihood that what was observed was an artifact of electrophoresis. In addition, when the HPLC purification procedure was repeated using the 35%-65% saturated ammonium sulfate fraction, containing the majority of spinach proteins, no immunoreactive protein bands were visible, demonstrating that the observation is not an artifact of the extraction or HPLC purification procedures. The presence of human serum albumin or its DNA in soil microorganisms has not been shown but cannot be entirely discounted as a source for potential uptake of protein or introduction of DNA into plants. However, the use of leaf tissue exclusively, and three different sources of plant material, including spinach grown on sterile growth media, reduces the possibility that the protein comes from soil microorganisms.

The high degree of sequence homology of human serum albumin with other mammalian albumins such as bovine serum albumin, and the presence of a possibly related protein in plants suggests that the NH_2-terminal amino acid sequence, of the protein described here, is conserved rather than an example of convergent evolution. While all the functions in plants have yet to be determined for the serum albumin-like protein, and the amino acid sequence reported represents only 15 of 585 residues, it is likely that its function is related to the properties of key conserved regions on both plant and human forms of the protein. Possible functions of the spinach protein, described here, might be based on the observed calcium, indole, and sugar binding that was demonstrated, however, more information about the cellular localization of the protein is required before a more precise functional model can be hypothesized.

Parallel transfer of a gene, which codes for serum albumin, from humans to plants or from plants to humans has not been shown and neither possibility can be entirely discounted. Alternately, the genetic evolution of the plant protein may have followed a similar path to primordial globins such as hemoglobin, which has been described in plants (Bogusz et al. 1988).

Fig. 3-1. **Plant proteins on Western blots probed with anti-calreticulin and anti human serum albumin antibodies.**

A, Western blot of white spruce (*Picea glauca* [(Moench) Voss]) needle proteins probed with polyclonal anti calreticulin antibody. *Lane 1*, recombinant calreticulin (3 μg), *Lanes 2-5*, white spruce (*Picea glauca* [(Moench) Voss]) microsomal membrane proteins (15 μg) B, Western blot of HPLC purified (*Lane 1*) spinach (*Spinacia oleracea* L.) proteins probed with anti human serum albumin antibody. Arrows indicate the position of two immunostained protein bands on the Western blot probed with polyclonal anti calreticulin antibody and a single immunostained band on the Western blot probed with polyclonal anti human serum albumin antibody. *Arrow a*, 70 kD band, *Arrow b*, 56 kD band.

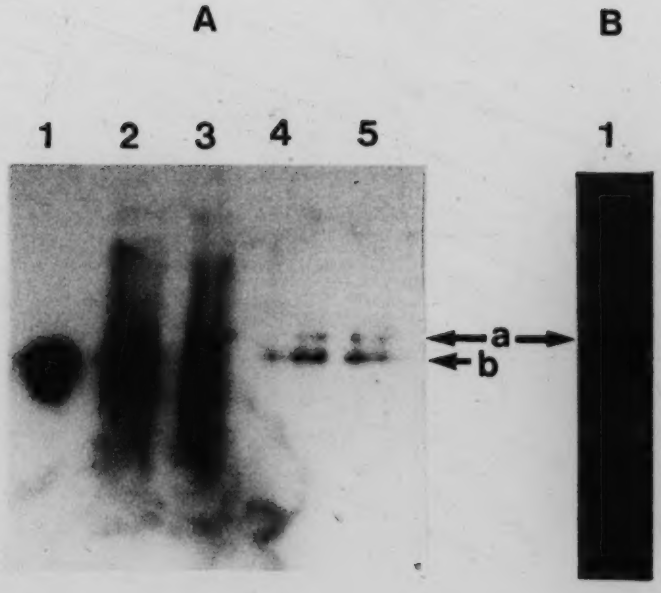

A
B
1 2 3 4 5
1
a
b

Fig. 3-2. **Cross-reactivity of calreticulin antibodies with human serum albumin.** A, Western blot probed with monoclonal anti human serum albumin antibody. *Lane 1*, human serum albumin (2 μg), *Lane 2*, HPLC fraction containing serum albumin-like plant protein purified from spinach (*Spinacia oleracea* L.) leaves (50 μg). B, Western blot probed with polyclonal anti calreticulin antibody. *Lane 1*, recombinant calreticulin (2 μg), *Lane 2*, canine calreticulin (2 μg), *Lane 3*, human serum albumin (2 μg), *Lane 4*, bovine serum albumin (2 μg). C, Duplicate Western blot (of B) probed with polyclonal anti human serum albumin antibody. *Lane 1*, recombinant calreticulin (2 μg), *Lane 2*, canine calreticulin (2 μg), *Lane 3*, human serum albumin (2 μg), *Lane 4* bovine serum albumin (2 μg).

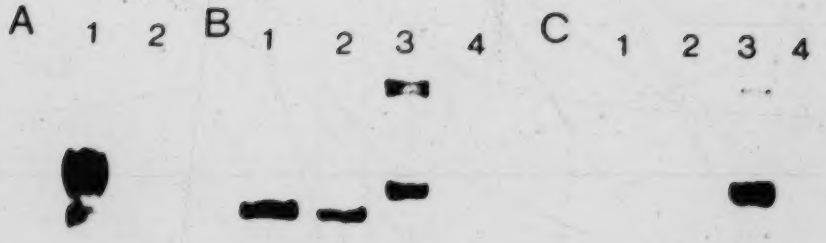

A 1 2 B 1 2 3 4 C 1 2 3 4

Fig. 3-3. **Purification of serum albumin-like protein from spinach (*Spinacia oleracea* L.) leaves using HPLC.** A, Elution profile of spinach (*Spinacia oleracea* L.) proteins from a Resource™ Q anion exchange HPLC column (6 mL), following selective ammonium sulfate precipitation. Sodium chloride concentrations were monitored over the elution profile and are shown. B, Immunoblots using HSA antisera were performed for pooled pairs of 1 mL HPLC fractions and are shown directly below the elution profile. Each lane contains approximately 20 μg of protein.

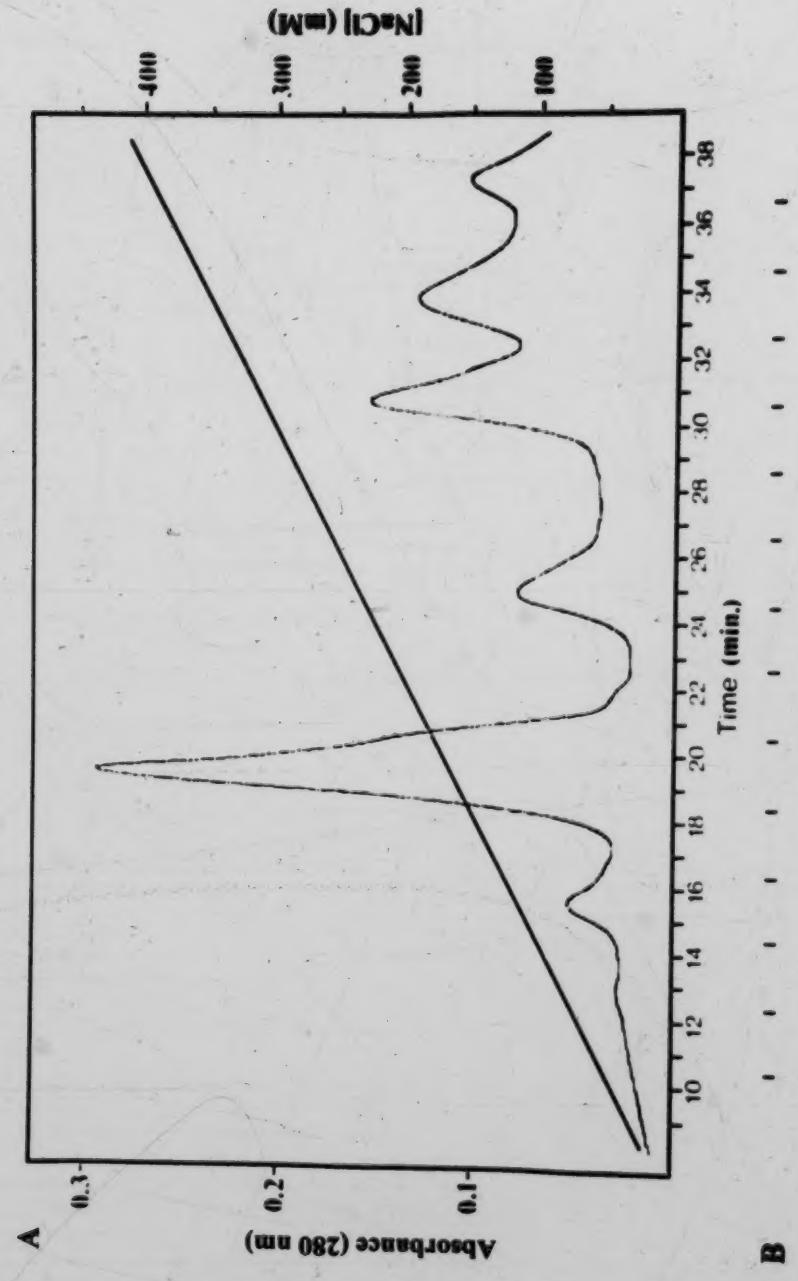

A
Absorbance (280 nm)
0.3
0.2
0.1
[NaCl] (mM)
400
300
200
100
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
Time (min.)
B

Fig. 3-4. **Two dimensional PAGE of HPLC purified spinach protein and comparison to HSA**. A, Coomassie blue and silver stained two dimensional PAGE gel of partially purified serum albumin-like spinach (*Spinacia oleracea* L.) protein from pooled Resource™ Q anion exchange HPLC fractions 24+25 (Fig. 3-3). B, Western blot of a two dimensional PAGE gel containing partially purified serum albumin-like spinach (*Spinacia oleracea* L.) protein from pooled HPLC fractions 24+25 (Fig. 3-3) and probed with human serum albumin (HSA) polyclonal antibodies. Total protein loaded on each of 2-D PAGE gels A and B was 20 μg. C, Two dimensional PAGE spotting pattern of 2 μg commercially purified human serum albumin (Sigma) blotted to nitrocellulose and probed with HSA polyclonal antibodies.

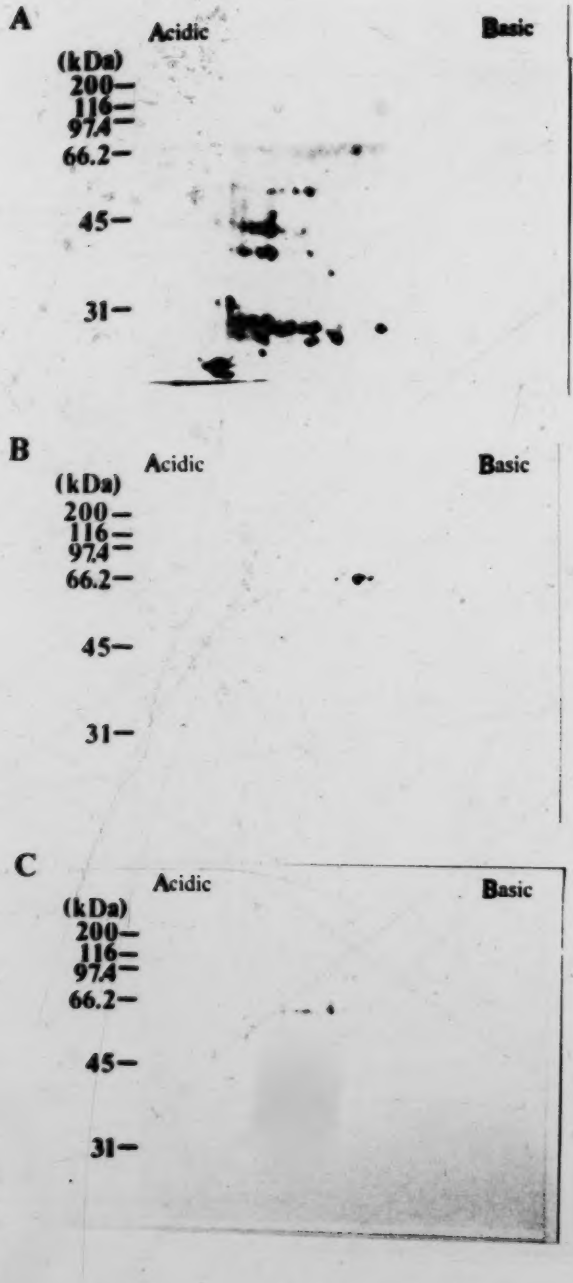

A
Acidic
Basic
(kDa)
200
116
97.4
66.2
45
31
B
Acidic
Basic
(kDa)
200
116
97.4
66.2
45
31
C
Acidic
Basic
(kDa)
200
116
97.4
66.2
45
31

Fig. 3-5. **Thymol and Stains-all staining of HPLC purified spinach (*Spinacia oleracea* L.) protein and ^{45}calcium overlay.** A, Coomassie Brilliant Blue R-250 staining, B, "Stains-all" staining, and C, Thymol-sulfuric acid staining of (2 μg) commercially purified human serum albumin (*lane 1*) and Resource™ Q HPLC purified spinach (*Spinacia oleracea* L.) proteins (10 μg) containing the serum albumin-like spinach protein (*lane 2*). Arrows point to the immunoreactive plant protein, D, $^{45}Ca^{2+}$ binding overlay performed on electroblot containing HPLC purified serum albumin-like spinach (*Spinacia oleracea* L.) protein. The nitrocellulose blotted fraction contains approximately 25 μg of protein. Arrow indicates the position of the radioactively labeled positive band corresponding to the immunoreactive plant protein..

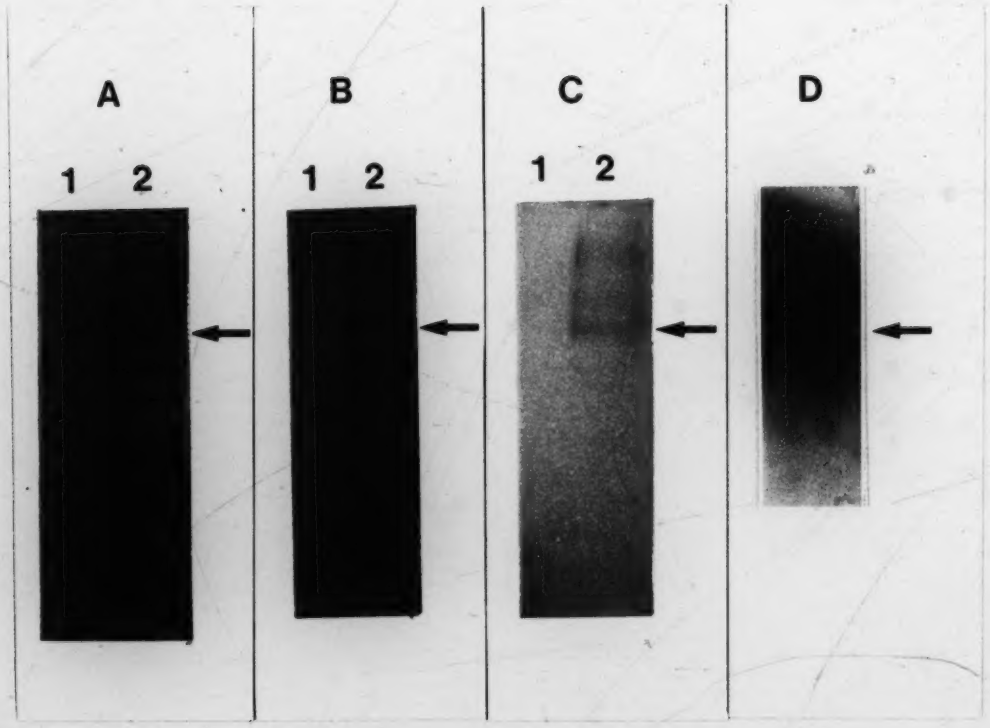
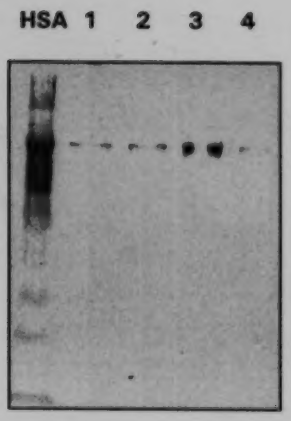

A
1 2
B
1 2
C
1 2
D

Fig. 3-6. **NH_2-terminal amino acid sequence alignment.** Shown are the NH_2-terminal amino acids of the 70 kD albumin-like protein from peas (*Pisum sativum* L.) and spinach (*Spinacia oleracea* L.) aligned with human serum albumin (HSA).

Peas:	F A H K S E H A S R
Spinach:	D A H K S E V A H R F K D L G
Human Serum Albumin:	D A H K S E V A H R F K D L G

Fig. 3-7. **Immunodetection of serum albumin-like protein in several plant species using polyclonal anti human serum albumin polyclonal antibody.** The proteins were purified by HPLC and electroblotted to nitrocellulose membrane. A, *Lane HSA*, human serum albumin (2 μg), *Lanes 1-4*, HPLC fractions containing purified protein from spinach (*Spinacia oleracea* L.) leaves (20 μg). B, *Lane HSA*, human serum albumin (2 μg), *Lanes 1-4*, HPLC fractions containing purified corn (*Zea mays* L.) protein (20 μg). C, *Lane HSA*, human serum albumin (2 μg), *Lane 1*, HPLC fraction containing purified protein from canola (*Brassica napus* L.) leaves (20 μg). D, *Lane HSA*, human serum albumin (2 μg), *Lanes 1-2*, HPLC fractions containing purified protein from Scots pine (*Pinus sylvestris* L.) leaves (20 μg).

A

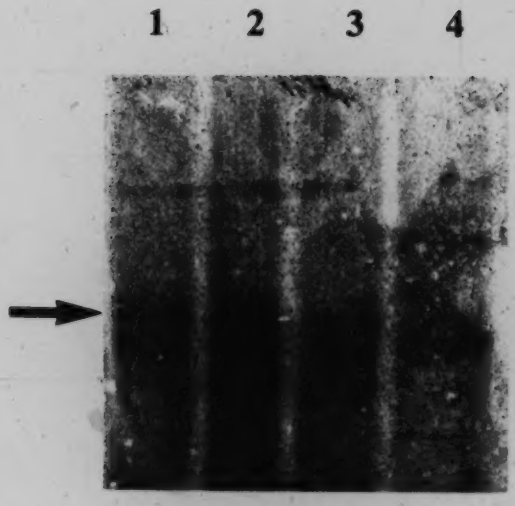


B

C

D

Fig. 3-8. **Auxin binding by HPLC purified serum albumin-like spinach (*Spinacia oleracea* L.) protein.** Proteins were purified by HPLC and prepared in vitro with 5 μM 5-[^{3}H]N_3IAA prior to electrophoresis as follows: *Lane 1* HPLC purified protein pH 7.0 control with no exogenous IAA. *Lane 2* HPLC purified protein pH 7.0 + 10 μM IAA. *Lane 3* HPLC purified protein pH 7.0 + 50 μM IAA. *Lane 4* HPLC purified protein pH 7.0 + 100 μM IAA.


1
2
3
4

3.5 References

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Chapter Four

4. Development of a purification strategy for the serum albumin-like protein from the shoots of peas (*Pisum sativum* L.) and leaves of spinach (*Spinacia oleracea* L).

4.1 Introduction

The purification of the plant protein which immunoreacts with human serum albumin antisera (described in Chapter 3) proved challenging using traditional purification techniques. Some non-specific interactions with chromatography columns and apparent hydrophobic characteristics, requiring special consideration, led to the development of a protocol for the purification of this novel plant protein. The purification in sufficient quantity and purity was required to allow further characterization of this protein. Basic approaches to protein purification are often combined with other techniques, such as the use of detergents, to combat problems encountered with other difficult to purify proteins (Vecchio et al. 1984; Bach et al. 1986; Shibata and Watanabe 1987; Kuwabara et al. 1988).

Column chromatography is widely used as a method of purifying proteins according to specific physical characteristics including surface charge, hydrophobicity, molecular size, and binding affinity to immobilized substrates. Crude extracts containing a mixture of different proteins are usually partially purified prior to application on chromatography columns, greatly increasing the purity and specificity of a protein purification strategy. Several techniques, including preparative isoelectric focusing with instruments such as Bio-Rad's Rotofor, various centrifugal filtration devices, specific

organelle isolation, and density gradient centrifugation of microsomal extracts, have been used to partially purify specific proteins. However, the most common means of preparing a protein extract for further purification procedures is selective ammonium sulfate precipitation.

Anion exchange columns for use with HPLC have improved the speed and reproducibility of chromatographic separations while reducing problems associated with dextran based open columns, including bed volume shrinkage at higher salt concentrations, voids or bubbles in the gel bed, and large bed volumes leading to sample dilution. The Resource™ Q anion exchange HPLC column (Pharmacia) is based on a quaternary ammonium group bonded to polystyrene/divinyl benzene beads. This HPLC column has the advantages of giving rapid separations of protein mixtures, high protein loading capacity, excellent reproducibility between separations, and very small void volumes with 1mL analytical columns.

The use of polystyrene bead based HPLC column supports is not without drawbacks when purifying proteins with hydrophobic properties. Cell wall protein and glycoprotein constituents of *Aspergillus fumigatus* have been shown to bind to polystyrene beads (Penalver et al. 1996). These glycoprotein and cell wall protein constituents were shown to account for the cell surface hydrophobic properties in *Aspergillus fumigatus* (Penalver et al. 1996).

As a result of problems encountered using various chromatography columns, a strategy for the purification the 70 kD serum albumin-like glycoprotein from plant extracts (described in Chapter 3) was developed. In the present study, selective

ammonium sulfate precipitation was used in combination with anion exchange column chromatography, and the zwitterionic detergent Triton X-100, to purify the plant protein. The 70 kD glycoprotein was partially purified in the 65%-85% saturated ammonium sulfate fraction during selective salting out steps and was subsequently purified by DEAE Sephadex A-50 anion exchange open column chromatography and Resource™ Q anion exchange HPLC.

4.2 Materials and Methods

4.2.1 Plant material

Protein used in HPLC purification procedures was extracted from commercially grown spinach leaves (*Spinacia oleracea* L.), locally outdoor grown spinach leaves (*Spinacia oleracea* L. cv. Tyee), and spinach (*Spinacia oleracea* L.) that was germinated on sterile growth media consisting of vermiculite and grown in a greenhouse. Protein used in DEAE Sephadex purification procedures was extracted from pea shoots (*Pisum sativum* L.) that were germinated on sterile vermiculite and grown in growth chambers and from the needles of greenhouse grown black spruce (*Picea mariana* [(Mill.) B.S.P.]) seedlings.

4.2.2 Selective ammonium sulfate precipitation

Plant tissues were homogenized in buffer containing 20 mM MOPS (pH 7.3) or 10 mM potassium phosphate (pH 7.1), 1 mM EGTA, 1 mM EDTA, 0.25 μM benzamidine, and 0.025 μM PMSF using a Waring blendor. The homogenate was filtered through 4 layers of cheesecloth and ammonium sulfate was added to 65% saturation (430 g/L). Following centrifugation at 14 300 x g, the supernatant was brought to 85% saturation by adding ammonium sulfate (150 g/L) and the pH was dropped to 4.5 using HCl. The solution was stirred for 2 hr. at 4 ^{0}C and centrifuged at 14 300 x g for 45 min. The resulting protein pellet was dissolved in a minimal volume of distilled water and the pH adjusted to 7.3. Dialysis was performed overnight against distilled water. For DEAE Sephadex A-50 open columns and Resource™ Q HPLC purification, with and without

0.5% (w/v) Triton X-100, ammonium sulfate precipitated proteins were lyophilized following dialysis and solubilized in column equilibration buffer consisting of 20 mM MOPS buffer (pH 7.3) or 10 mM potassium phosphate buffer (pH 7.1), containing 0.5 mM DTT, 1 mM EGTA, and 1 mM EDTA with and without 0.5% (w/v) Triton X-100.

4.2.3 Anion exchange chromatography with DEAE Sephadex A-50

Open ended glass DEAE Sephadex A-50 column beds (250 mm X 25 mm) were prepared in column equilibration buffer systems as follows: For pea and black spruce protein separations, 10 mM potassium phosphate, pH 7.1 containing 0.5 mM DTT, 1 mM EGTA, and 1 mM EDTA was used to equilibrate columns. Spinach protein separations used column equilibration buffer consisting of 20 mM MOPS, pH 7.3 containing 0.5 mM DTT, 1 mM EGTA, and 1 mM EDTA with and, for some separations, without 0.5% (w/v) Triton X-100.

Following ammonium sulfate precipitation, dialysis and lyophilization, spinach proteins were re-suspended in a minimal volume of column equilibration buffer consisting of 20 mM MOPS, pH 7.3 containing 0.5 mM DTT, 1 mM EGTA, and 1 mM EDTA.

Following ammonium sulfate precipitation, dialysis, and lyophilization, pea and black spruce proteins were resuspended in a minimal volume of column equilibration buffer consisting of 10 mM potassium phosphate, pH 7.1 containing 0.5 mM DTT, 1 mM EGTA, and 1 mM EDTA.

Following the application of protein samples, columns were washed with five bed volumes of equilibration buffer. An HPLC pump generated continuous gradient of NaCl

in equilibration buffer from 10 mM to 800 mM in 200 minutes at a flow rate of 2.0 mL min^{-1} was used to elute proteins from the DEAE Sephadex A-50 columns. Fractions were collected (5 mL) and prepared for analysis by electrophoresis, western blotting, and immunodetection.

4.2.4 Resource™ Q anion exchange HPLC

Dried protein pellets, treated with organic solvent phase extraction (Pohl 1990) or non-phase extracted, were dissolved in 20 mM MOPS buffer, pH 7.3 containing 0.5 mM DTT, 1 mM EGTA, and 1 mM EDTA. Proteins were separated by anion exchange HPLC using a 6 mL Resource™ Q anion exchange column (Pharmacia LKB Biotechnology Baie D'Urfe, PQ, Canada). Buffers used to elute proteins were: Buffer A 20 mM MOPS, pH 7.3, containing 0.5 mM DTT, 1 mM EGTA, and 1 mM EDTA; Buffer B 20 mM MOPS, pH 7.3, containing 0.5 mM DTT, 1 mM EGTA, 1 mM EDTA and 1 M NaCl. The following gradient was used to elute proteins: time 0-6 min, 0-1% buffer B; time 6-36 min, 1-50% buffer B, flow rate of 1 mL min^{-1}. Fractions (1 mL) were collected using the Bio-Rad 2110 fraction collector (Bio-Rad Laboratories, Mississauga, ON, Canada) and prepared for electrophoresis. For 1 mL Resource™ Q anion exchange columns (Pharmacia LKB Biotechnology) the following buffers were used to elute proteins: 20 mM MOPS, pH 7.0, containing 0.5 mM DTT (buffer A) and 20 mM MOPS, pH 7.0, containing 0.5 mM DTT, 1 M NaCl (buffer B). The following gradient was run to separate proteins: time 0-2 min, 0-1% buffer B, 2-20 min, 1-50% buffer B, with the flow rate of 1 mL min^{-1}. Fractions

(1 mL) were collected with the Bio-Rad 2110 fraction collector (Bio-Rad Laboratories, Mississauga, ON, Canada) and processed for electrophoresis.

4.2.5 Resource™ Q anion exchange HPLC with Triton X-100

Lyophilized protein samples containing approximately 15 mg protein were dissolved in 0.5 ml of 20 mM MOPS buffer pH 7.0 containing 0.5 mM DTT with 0.5% (w/v) Triton X-100 and separated by Resource™ Q anion exchange HPLC (Pharmacia LKB Biotechnology, Baie D'Urfe, PQ, Canada). Anion exchange HPLC was carried out using a 1 mL Resource™ Q anion exchange column (Pharmacia LKB Biotechnology) with the following buffers used to elute proteins: 20 mM MOPS, pH 7.0, containing 0.5 mM DTT with 0.5% (w/v) Triton X-100 (buffer A) and 20 mM MOPS, pH 7.0, containing 0.5 mM DTT, 1 M NaCl with 0.5% (w/v) Triton X-100 (buffer B). The following gradient was run to separate proteins: time 0-2 min, 0-1% buffer B, 2-20 min, 1-50% buffer B, with the flow rate of 1 mL min^{-1}. Fractions (1 mL) were collected with the Bio-Rad 2110 fraction collector (Bio-Rad Laboratories, Mississauga, ON, Canada) and processed for electrophoresis.

4.2.6 Sephadex G200 gel filtration chromatography

Gel filtration chromatography was performed on a protein sample obtained by selective ammonium sulfate precipitation which was dialyzed and freeze-dried, then dissolved in sample buffer consisting of 20 mM MOPS, 0.5 mM DTT and 0.5 % (w/v) Triton X-100. The sample was applied to a 25 mm x 300 mm column of Sephadex G-200

and eluted with the sample buffer. Fractions (3 mL) were collected and processed for electrophoresis.

4.2.7 Organic solvent extraction of crude extracts before HPLC

For purifications using organic solvent treated ammonium sulfate precipitated extracts, proteins were precipitated and defatted using a chloroform/methanol/water, 1/4/3 (by volume) phase mixture as described by Pohl (1990). The phase mixture was vortexed and centrifuged briefly at 1500 x g to separate the phases. The upper phase was discarded, leaving the protein at the interface, and 3 volumes of methanol were added to the remaining chloroform phase. After centrifugation at 1500 x g for 15 min, the protein pellet was recovered and dried under vacuum. Dried protein was dissolved in 2.5 mL of 20 mM MOPS buffer, pH 7.3 containing 0.5 mM DTT, 1 mM EGTA, and 1 mM EDTA.

4.2.8 Electrophoresis, electroblotting, and immunodetection

One dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed as described by Laemmli (1970) with a 10% acrylamide resolving gel using the Bio-Rad Mini-Protean II apparatus. Proteins were precipitated using a chloroform/methanol/water system as described by Pohl (1990) or a mixture of ice cold ethanol: acetone 1:1 (v/v).

Proteins used for SDS PAGE were dissolved in 1-D PAGE sample buffer containing 60 mM Tris-HCl (pH 6.8), 4% SDS, 0.1 M DTT, 10% 2-mercaptoethanol,

20% glycerol and 0.01% bromophenol blue. All samples were loaded on gels using sterile, disposable gel loader pipet tips (Bio-Rad Laboratories).

Following electrophoresis, gels were either electroblotted to nitrocellulose (Towbin et al. 1979) or stained with coomassie blue, or silver. Nitrocellulose membranes were blocked overnight in Tris-buffered saline containing 5% (w/v) BLOTTO (non-fat milk powder).

Proteins transferred to nitrocellulose membranes were probed with primary polyclonal antibody (1:500) raised in goat against human serum albumin (Sigma Chemical Company). Secondary antibody (1:3000) conjugated to horseradish peroxidase was used to label immunoreactive bands for visualization using enhanced chemiluminescence (Amersham Life Sciences, Oakville, ON, Canada) or antibody (1:5000) conjugated to alkaline phosphatase for visualization using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) in 100 mM Tris buffer pH 9.5 containing 100 mM NaCl, 5 mM $MgCl_2$. Antibodies were diluted in Tris-buffered saline containing 1% (w/v) BLOTTO (non-fat milk powder) and 0.05% Tween 20 (TTBS).

Protein quantity estimation was performed using the bicinchoninic acid (BCA) protein assay (Pierce Chemical).

4.2.9 Chemicals and supplies

All chemicals were of the highest grade available and were purchased from Sigma Chemical Company and Fisher Scientific. Nitrocellulose membrane was purchased from Micron Separations Inc., (Westboro, MA, USA). Prestained molecular weight standards

were obtained from Bio-Rad Laboratories and Sigma Chemical Company. Resource™ Q HPLC columns (1 mL and 6 mL) were obtained from Pharmacia LKB Biotechnology (Baie D'Urfe, PQ, Canada). Anti-calreticulin antibody was supplied by Dr. M. Michalak (Department of Biochemistry, University of Alberta, Edmonton, Canada).

4.3 Results

4.3.1 Selective ammonium sulfate precipitation

The serum albumin-like plant protein was selectively salted out in the 65%-85% saturated ammonium sulfate fraction. The 35%-50% and 50%-65% saturated ammonium sulfate fractions yielded no proteins detectable using the anti-HSA, or HSA cross-reacting calreticulin, polyclonal antibodies (results not shown).

4.3.2 DEAE Sephadex A-50 chromatography

Proteins in crude homogenates from peas, black spruce, and spinach were precipitated using 65%-85% saturated ammonium sulfate and were separated on DEAE Sephadex A-50 open columns. Fig. 4-1A shows a profile of the measured absorbance at 280 nm for a DEAE Sephadex A-50 column separation of pea proteins. Following SDS PAGE on 10% gels, proteins were electroblotted to nitrocellulose. Antibodies raised against vertebrate calreticulin, which cross-reacted with serum albumin (see Chapter 3), recognized protein bands of 60 kD and 70 kD in fractions throughout the elution profile (Fig. 4-1B). Human serum albumin antibodies also recognized a 70 kD plant protein band that was present in most of the fractions of the elution profile (Fig. 4-1C). Elution buffers containing 0.5% (w/v) Triton X-100 improved the separation of proteins in the presence of interfering colored substances, which were presumed to be tannins or phenolic compounds, and allowed elution of immunoreactive protein bands with 280-300 mM NaCl.

4.3.3 Resource™ Q anion exchange HPLC

Ammonium sulfate precipitated spinach proteins from the 65%-85% saturated fraction were separated using a Resource™ Q anion exchange HPLC column. However, a similar problem to what was encountered using DEAE Sephadex A-50 affected the protein separation. The 70 kD serum albumin-like protein from crude extracts contained colored substances that appeared to bind non-specifically with the anion exchange HPLC column and subsequently appeared in many fractions throughout the elution profile (Fig. 4-2). Two procedures allowed the isolation of the albumin-like protein into only a few HPLC fractions:

Using a chloroform/methanol/water (1/4/3) phase system as described by Pohl (1990) to treat ammonium sulfate precipitated extracts, prior to HPLC purification, colored substances were removed and protein separations improved significantly (Fig. 4-5). The 70 kD albumin-like protein was eluted from the anion exchange column with 200-250 mM NaCl when solvent phase extraction (Pohl 1990) was used on the ammonium sulfate precipitated proteins prior to HPLC separation.

The second method which improved HPLC separation of ammonium sulfate precipitated proteins in the presence of colored substances involved the use of 0.5% (w/v) Triton X-100 in the elution buffers.

4.3.4 Resource™ Q anion exchange HPLC with Triton X-100

Adding 0.5% (w/v) Triton X-100 to the elution buffers used for Resource™ Q anion exchange HPLC, improved separation of the serum albumin-like plant protein into only a few fractions. However, when HPLC fractions were screened with serum albumin cross-reacting calreticulin antibody (see Chapter 3), a large, 100-150 kD, diffuse immunoreactive band was observed in the HPLC fractions containing the serum albumin-like protein (Fig. 4-3B). The protein, exhibited anomalously high molecular weight and eluted in only a few fractions of the salt gradient corresponding to 200-250 mM NaCl.

Ammonium sulfate precipitated proteins were separated by gel filtration using Sephadex G-200 with 0.5% (w/v) Triton X-100 in the elution buffers and all fractions were screened with the serum albumin cross-reacting anti calreticulin antibody. Following SDS PAGE, an immunoreactive diffuse band was found in the 100-150 kD fractions, indicating the formation of high molecular weight aggregates in the presence of Triton X-100 (Fig. 4-4A).

The fractions which were pooled from the anion exchange HPLC separation were dialysed against distilled water, precipitated with acetone:ethanol (1:1) and washed 3 times with 95% ethanol to remove Triton X-100 prior to separation by SDS PAGE. Using this washing procedure, most of the Triton X-100 was effectively removed from the studied protein. The wash treatment resulted in lowering of the apparent molecular weight and separation of the large diffuse band into one clearly immunoreactive band with a slight smear below it (Fig. 4-4B).

4.3.5 Organic solvent extraction of crude extracts

Organic solvent extraction (Pohl 1990) of crude plant homogenates and ammonium sulfate precipitated proteins prior to column chromatography resulted in superior chromatographic separations (Fig. 4-5) and less streaking in lanes of SDS PAGE gels. Chlorophyll partitioned into the lower (chloroform) phase and yellow- or brown-colored substances partitioned into the upper phase (methanol/water) of the two phase system.

4.4 Discussion

The purification of the serum albumin-like protein from plants posed some challenging problems. The protein appeared to bind non-specifically to several column matrices as well as exhibited mixed ionic and hydrophobic characteristics. When crude protein extracts were purified by "salting out" with ammonium sulfate, colored substances co-purified with target proteins and when ion exchange columns were used to further purify extracts, these colored substances appeared in many different fractions over the entire elution profile. The colored substances, which interfered with subsequent analytical steps including SDS PAGE, were tentatively identified as tannins or other phenolic compounds, which are abundant in plants and have been suggested to form associations with proteins (Dawra et al. 1988; Baxter et al. 1997; Zhu et al. 1997). It is possible that binding of polyphenolics and tannins may affect surface properties such as charge and hydrophobicity. The glycosylation of the serum albumin-like plant protein (see Chapter 3) may also contribute to the observed hydrophobic properties in a similar way to what was observed for the cell wall protein and glycoprotein constituents of *Aspergillus fumigatus* (Penalver et al. 1996). The simplest way to remove the colored substances from the plant protein extracts, prior to chromatographic separation, was by using a chloroform/methanol/water phase system as described by Pohl (1990). Conifer needles contain a number of terpenoids which in addition to polyphenolics that can interfere with protein assays and electrophoresis. A similar application of organic solvents to remove substances which interfere with protein analysis in plants has been shown to improve protein separations from conifer needle tissue prior to SDS PAGE (Ekramoddoullah 1993).

Unfortunately, the use of organic solvent treatments leads to protein denaturation and may cause some loss of biological activity through the loss of bound ligands including tannins as suggested by Zhu et al. (1997). Serum albumins have been shown to bind strongly to tannins (Tsarevskii and Karal'nik 1975; Dawra et al. 1988) which may explain why the serum albumin-like plant protein co-chromatographs with the yellow colored tannin-like substances.

The zwitterionic detergent Triton X-100 improved chromatographic separations of the serum albumin-like protein when anion exchange chromatography columns were used. Triton X-100 extraction of proteins has previously been used in combination with Sephadex A-50 columns by other researchers to improve separations of some proteins. (Shibata and Watanabe 1987; Kuwabara et al. 1988). However, in this study, the use of Triton X-100 during purification resulted in high molecular weight immunoreactive complexes of the serum albumin-like protein. These complexes were not broken down during SDS PAGE. The problem of high molecular weight complexes was observed when open DEAE Sephadex A-50 columns and Resource™ Q anion exchange HPLC columns were used in combination with Triton X-100. Results obtained using Sephadex G200 gel filtration columns confirmed the formation of these immunoreactive high molecular weight complexes, of approximately 100-150 kD, when Triton X-100 was used in the elution of ammonium sulfate fractionated proteins.

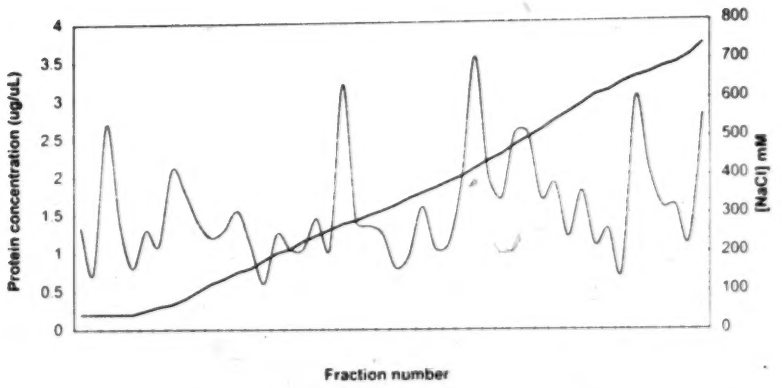
High molecular weight complexes and altered enzyme properties have been previously reported by other researchers who used Triton X-100 in protein purification strategies. Katona et al. (1992) reported the formation of protein aggregates, in the

presence of Triton X-100, which were stable to SDS PAGE. Piemonte et al. (1993) reported the formation of large aggregates and increased activity of glutathione S-transferase in the presence of 1% Triton X-100. Large detergent-protein complexes were observed by Hutchison and Fox (1989) when they used Triton X-100 in combination with gel filtration chromatography to purify a placental protein. A dense, diffuse, protein band and a less dense narrow band were observed when Triton X-100 was used in the purification of an aminopeptidase (Shibata and Watanabe 1987). By using Triton X-100 with DEAE Sephadex A-50 column chromatography, Kuwabara et al. (1988) observed a single broad protein band when the protein that they purified was subjected to SDS PAGE. Repeated 95% ethanol washing steps could reduce or eliminate the large protein-detergent complexes that were observed when Triton X-100 was used in chromatographic purification of the spinach serum albumin-like protein. Organic solvent extraction using the chloroform/methanol/water (1/4/3, by volume) method of Pohl (1990) prior to column chromatography eliminated the need for difficult removal of detergents and other interfering substances prior to subsequent protein analysis. However, the improved chromatographic separation of organic solvent-treated proteins was achieved with the drawback of protein denaturation.

In summary, this chapter describes a multiple step purification strategy that employed selective ammonium sulfate precipitation and anion exchange chromatography for the rapid and reproducible purification of the plant serum albumin-like protein that was described in Chapter 3.

Fig. 4-1. **Anion exchange DEAE Sephadex A-50 purification of the serum albumin-like protein from pea (*Pisum sativum* L.) shoots.** A, Elution profile of proteins from a DEAE Sephadex A-50 anion exchange column, following selective ammonium sulfate precipitation. Sodium chloride concentrations are shown with the elution profile. B, Immunoblots, using HSA cross-reacting anti calreticulin antibodies, of column fractions encompassing the entire elution profile shown in A. C, Immunoblots, using anti human serum albumin antibodies, of column fractions encompassing the entire elution profile shown in A. Each lane contains 10-15 μg of protein.

A


Protein concentration (ug/uL)
4
3.5
3
2.5
2
1.5
1
0.5
0
800
700
600
500
400
300
200
100
0
[NaCl] mM
Fraction number

B

C

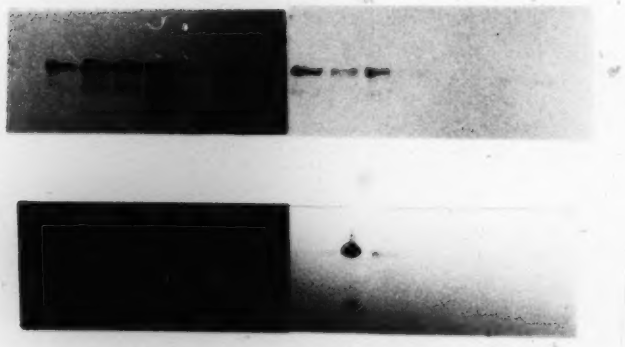
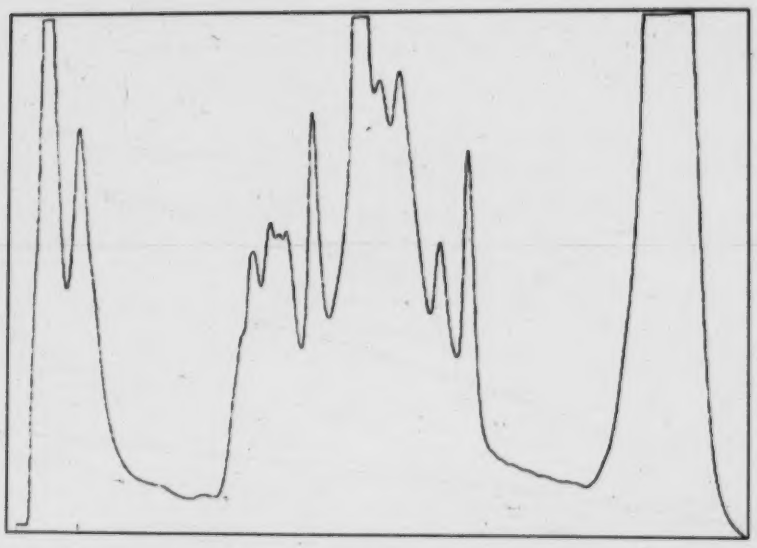


Fig. 4-2. **Resource™ Q HPLC purification of the serum albumin-like protein from spinach (*Spinacia oleracea* L.) leaves using a 1 mL analytical column.** A, Elution profile of proteins from a Resource™ Q anion exchange HPLC column (1 mL column volume), following selective ammonium sulfate precipitation. Sodium chloride gradients were as described for 1 mL Resource™ Q anion exchange HPLC columns. B, Immunoblots using HSA antisera were performed for pooled pairs of 1 mL fractions encompassing the elution profile shown. Each lane contains approximately 20 μg of protein.

A


0 2 4 6 8 10 12 14 16 18 20 22
Time (min) / Fraction number

B

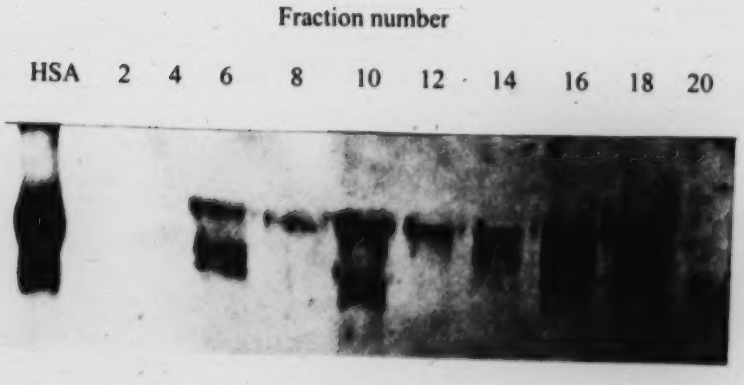
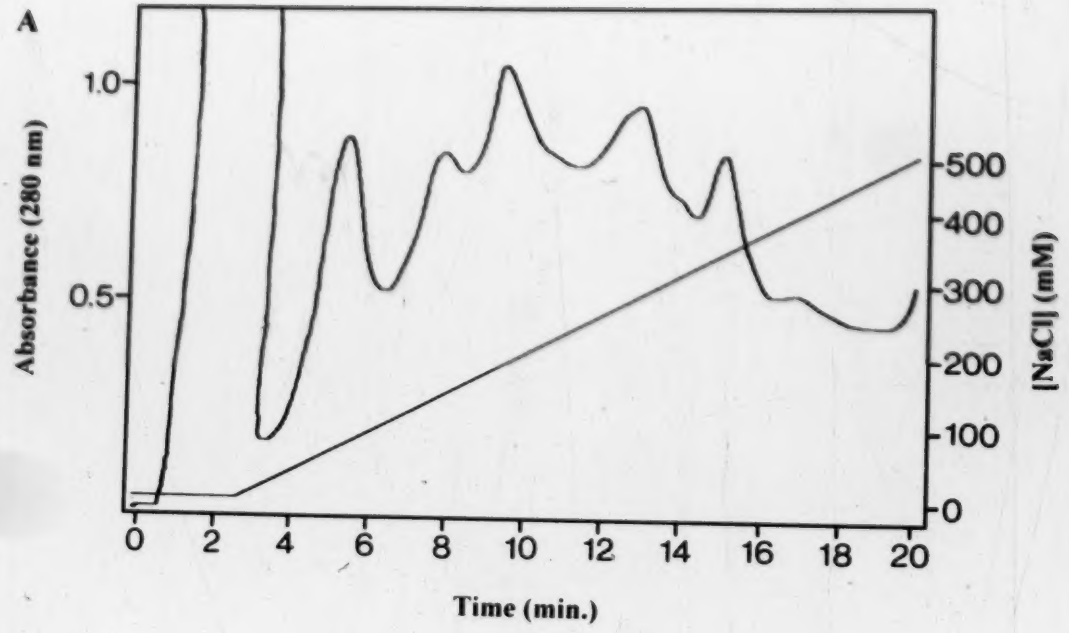

Fraction number
HSA 2 4 6 8 10 12 14 16 18 20

Fig. 4-3. **Resource™ Q HPLC purification of the serum albumin-like protein from spinach (*Spinacia oleracea* L.) leaves using a 1 mL analytical column with Triton X-100 included in elution buffers.** A, Elution profile of proteins from a Resource™ Q anion exchange HPLC column (1 mL column volume), following selective ammonium sulfate precipitation. Sodium chloride concentrations for the elution profile are shown. B, Immunoblots using HSA antisera were performed for the selected 1 mL fractions shown. Each lane contains approximately 20 μg of protein.


A
Absorbance (280 nm)
1.0
0.5
0
2
4
6
8
10
12
14
16
18
20
Time (min.)
500
400
300
200
100
0
[NaCl] (mM)

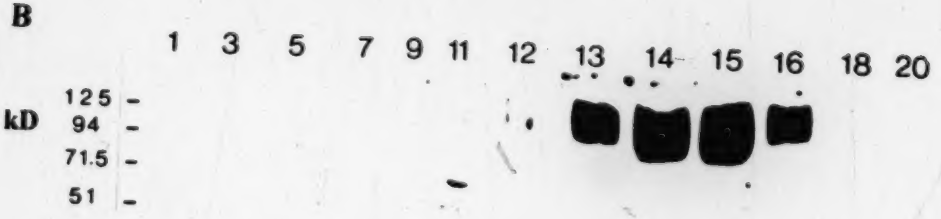

B
1
3
5
7
9
11
12
13
14
15
16
18
20
kD
125
94
71.5
51

Fig. 4-4. **Sephadex G200 SEC chromatography of ammonium sulfate precipitated spinach (*Spinacia oleracea* L.) proteins in the presence of Triton X-100.** A, Ammonium sulfate precipitated proteins were separated on a Sephadex G-200 column with Triton X-100 in the elution buffers, and were immunoblotted using human serum albumin cross-reacting calreticulin antibodies. *Lanes 11-17,* correspond to the 100-200 kD fractions (3 mL each) containing 50 μg of protein. B, Protein purified by HPLC in the presence of Triton X-100 shown after washing step with 95% ethanol.

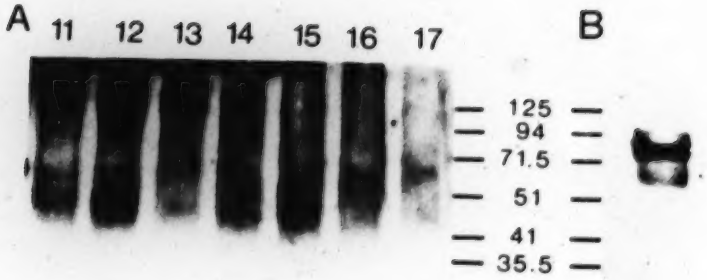
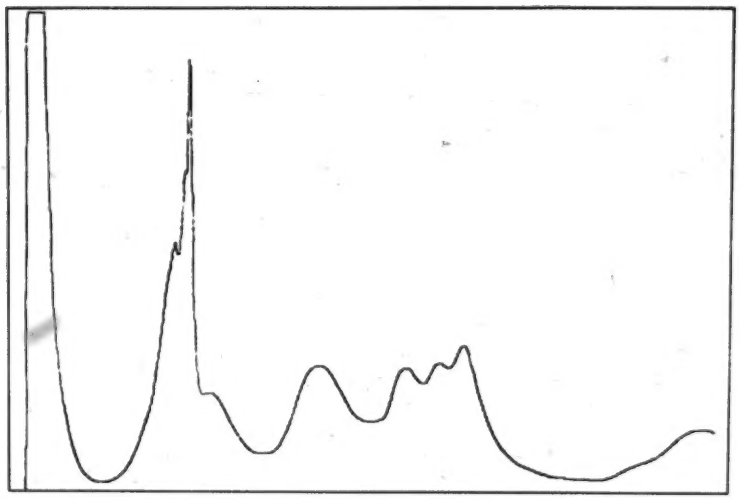
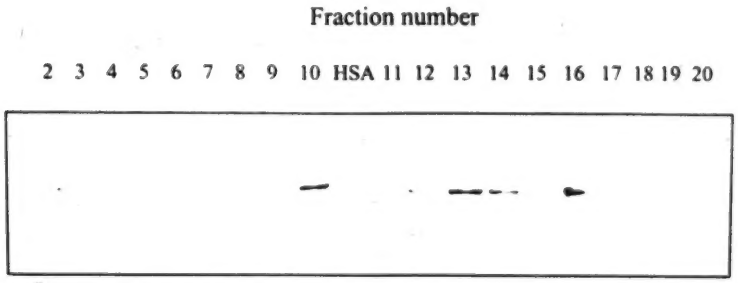

A
11
12
13
14
15
16
17
B
125
94
71.5
51
41
35.5

Fig. 4-5. **Resource™ Q HPLC purification following solvent phase extraction of ammonium sulfate precipitated spinach (*Spinacia oleracea* L.) proteins.** A, Elution profile of proteins from a Resource™ Q anion exchange HPLC column (1 mL column volume), following selective ammonium sulfate precipitation and organic solvent phase extraction (Pohl 1990). Sodium chloride gradients were as described for 1 mL Resource™ Q anion exchange HPLC columns. B, Immunoblots using HSA antisera were performed for pooled pairs of 1 mL fractions encompassing the elution profile shown. Each lane contains approximately 20 μg of protein.

A


0 2 4 6 8 10 12 14 16 18 20
Time (min) / Fraction number

B


Fraction number
2 3 4 5 6 7 8 9 10 HSA 11 12 13 14 15 16 17 18 19 20

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Chapter Five

5. Cellular immunolocalization of a 70 kD serum albumin-like protein in *Spinacia oleracea* L. leaves.

5.1 Introduction

In order to elucidate the functions of the serum albumin-like spinach (*Spinacia oleracea* L.) protein described in this dissertation and determine the degree to which its functions might be similar to human serum albumin, it was necessary to identify where the plant protein was transported within cells following its synthesis.

Human serum albumin was localized to the secretory pathway, where it is synthesized in the human liver and is secreted via the Golgi bodies into the bloodstream (Brown and Schockley 1982).

The Golgi of plants functions as an organelle that compartmentalizes cellular products into vesicles which may then be emptied intracellularly to vacuoles or extracellularly (Chrispeels 1991). The Golgi is a major site of post translational protein modification which may include the attachment of sugars (De Vries et al. 1988). Glycosylation is a common feature of vacuolar and extracellular proteins that pass through the Golgi complex (De Vries et al. 1988).

Several ligand binding sites within the human serum albumin protein have been reported and are described in Chapter 2. The ligand binding sites of human serum albumin, which are of potential interest in determining the functions of the serum

albumin-like protein in plants, include those for the binding of phenolic compounds, indole derivatives, calcium, and fatty acids (Geisow and Beaven 1977; McMenamy 1977; Spector and Fletcher 1978; Dawra et al. 1988).

The serum albumin-like plant protein was initially identified in microsomal membrane preparations from plants using polyclonal antibodies raised against human serum albumin (Chapter 3). The serum albumin-like protein was purified from spinach leaves and some of its properties were determined. The plant protein shared significant amino acid sequence similarity with the human protein at the NH_2-terminus, however, unlike human serum albumin (Brennan et al. 1990), the plant protein was glycosylated and was associated with galacturonic acid, a component of cell wall polysaccharides. The region of conserved amino acid sequence between the plant protein and human serum albumin was previously described as containing binding sites for calcium, indole derivatives, and nickel in humans (McMenamy 1977; Geisow and Beaven 1977; Zhou et al. 1994). Calcium and indole binding by the HPLC purified plant serum albumin-like protein was shown in Chapter 3.

The chemical properties (Chapter 3) and hypothetical functions described for serum albumins in mammals suggest that the serum albumin-like plant protein might serve a role as a transporter of components required for cell wall synthesis. Therefore, in this study, cell fractionation, continuous sucrose density gradient fractionation of leaf microsomal membranes, and tissue printing were used in conjunction with membrane enzyme marker assays and polyclonal antibodies raised against human serum albumin, to localize the serum albumin-like protein in plant cells.

5.2 Materials and Methods

5.2.1 Plant material

Plant homogenates used for the isolation of microsomal membranes and chloroplasts were prepared from commercially-grown spinach (*Spinacia oleracea* L.) leaves. Plant material used for tissue printing was excised from the petioles of commercially-grown spinach and indoor-grown, African violet plants (*Saintpaulia ionantha*). The petioles of African violets (*Saintpaulia ionantha*) were found to give excellent tissue imprints on nitrocellulose.

5.2.2 Microsomal membrane isolation

Spinach leaves (25 g) were homogenized 8 x 30 sec. at 24 000 rpm with an IKA homogenizer (IKA Werke Janke & Kunkel GmbH & Co KG, Staufen, Germany) in 200 mL of 0.75 M Tris-acetate buffer, pH 7.0, containing 7.5% (w/v) polyvinylpolypyrrolidone (PVPP), 0.25 M sucrose, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.15% 2-mercaptoethanol. The slurry was filtered through 4 layers of cheesecloth and the filtrate was centrifuged in a Beckman SW 28 rotor for 20 min. at 4 300 x g. The pellet was discarded and the supernatant was centrifuged for 1 hr. at 75 000 x g. The resulting microsomal pellet and supernatant were prepared for electrophoresis.

For fractionation of microsomal membrane proteins with Triton X-114, freshly isolated microsomal membranes from spinach leaves were resuspended in distilled water and used immediately.

Protein content in both microsomal and non-membrane protein fractions was determined by the BCA protein assay (Pierce Life Science & Analytical Research Products).

5.2.3 Triton X-114 fractionation of microsomal membranes

Spinach leaf microsomes were subjected to Triton X-114 fractionation as described by Kjellbom et al. (1989). Membrane vesicles were suspended in distilled water at 2 mg of protein per mL and mixed with an equal volume of Triton X-114 solution resulting in a final composition of 1% Triton X-114, 150 mM NaCl, 1 mM EDTA and 10 mM Tris-HCl (pH 7.6). The mixture was vortexed and incubated for 10 min. at 0 ^{0}C followed by additional vortexing and incubation for 5 min. at 0 ^{0}C. The mixture was warmed to 37 ^{0}C in order to separate into a detergent-enriched lower phase and a detergent depleted upper phase. After 15 min. of incubation, the mixture was centrifuged at 500 x g for 5 min. The upper phase was repartitioned after bringing the concentration of Triton X-114 to 1% and the lower phase was repartitioned with fresh medium containing no Triton X-114. Lower and upper phases were collected from the re-partitioned samples and prepared for electrophoresis.

5.2.4 Chloroplast isolation

Fresh spinach leaves (10 g) were harvested, cut into 2 mm wide strips, and soaked for 5 minutes in ice-cold homogenization buffer consisting of 30 mM Tris-HCl pH 7.8 and 0.33 M sucrose. Leaves were homogenized using an IKA Ultra Turrax homogenizer (IKA Werke Janke & Kunkel GmbH & Co KG, Staufen, Germany) for 2 X 20 seconds at 24 000 RPM. The homogenate was filtered through four layers of cheesecloth and the

filtrate was centrifuged for 1 minute at 100 X g to collect cell debris. The supernatant was centrifuged for 5 minutes at 1500 X g to pellet chloroplasts. The chloroplast pellet was resuspended in a minimal volume of homogenization buffer and 5 mL of the suspension was layered on a 20 mL gradient consisting of 10 mL of 35% (w/v) sucrose over 10 mL of 70% (w/v) sucrose, each prepared in homogenization buffer. The gradients were centrifuged for 20 minutes at 3000 X g and the chloroplasts were recovered from the 35%-70% interface. Chloroplasts were resuspended in 20 mL of homogenization buffer and pelleted by centrifugation at 10 000 X g for 10 minutes. Chloroplast purity was monitored by microscopic examination.

5.2.5 Sucrose density gradients

Continuous, linear gradients of 37 mL ranging from 20% to 45% (w/w) sucrose in 1 mM $MgSO_4$ and 1 mM Tris-MES, pH 7.2 were formed, in polyalomar tubes designated for the Beckman SW 28 rotor, using a dual pump HPLC (LKB).

Freshly isolated microsome pellets were resuspended in 18% sucrosé suspension media prepared in 1 mM Tris-MES containing 1 mM $MgSO_4$. A total suspended microsome volume of 1.5 mL containing approximately 10-15 mg protein was carefully layered onto the top of linear sucrose gradients, formed during the preparation of the microsomal fraction. The gradients were centrifuged at 100 000 x g for 3.5 hours using a Beckman SW 28 rotor.

Following centrifugation, gradient contents were collected in 1.5 mL fractions by puncturing the bottom of the centrifuge tube. Densities of fractions were determined for 1 mL samples and all fractions of equal density were pooled for further analysis.

5.2.6 Membrane marker enzyme assays

5.2.6.1 Cytochrome c oxidase (mitochondrial marker)

Cytochrome c oxidase was performed as for NAD(P)H -cytochrome c reductase except that the oxidation of reduced cytochrome c was observed. Cytochrome c was reduced by the addition of excess sodium dithionite. Aeration for 5 minutes removed excess dithionite. Membrane suspension (0.1 mL) was added to a reaction mixture containing 0.1 mL of 0.3% digitonin or Triton X-100 and 2.7 mL of 50 mM sodium phosphate pH 7.5. The assay reaction was started by adding 0.1 mL of reduced cytochrome c, and the change in O.D. $_{550}$ was monitored. Calculation of oxidation rate used an extinction coefficient of 18.5 mM^{-1} cm^{-1} (Hodges and Leonard 1974).

5.2.6.2 Cytochrome c reductases (ER and tonoplast markers)

NADH-cytochrome c reductase marker enzyme assay was performed by the method of Hatefi and Rieske (1967). NADPH-cytochrome c reductase marker enzyme assay was performed by the method of Masters et al. (1967). Assays were performed at room temperature (25 ^{0}C) and reduction of cytochrome c was measured using an LKB spectrophotometer set at λ=550 nm. Each reaction included 0.1 mL of enzyme (sucrose gradient fraction), 0.1 mL of 50 mM sodium cyanide, 0.2 mL of 0.45 mM cytochrome c, and 2.5 mL of 50 mM phosphate buffer pH=7.5. Reactions were started by adding 0.1 mL of either 3 mM NADH or NADPH. Initial rates of cytochrome c reduction and an extinction coefficient of 18.5 mM^{-1} cm^{-1} were used to calculate reductase activity.

5.2.6.3 IDPase (Golgi marker)

Inosine diphosphatase (IDPase) activity of sucrose gradient fractions was determined using the method described by Gillott et al. (1980) with the following modifications: Sucrose gradient fractions of equal density were pooled and diluted using 0.025 M Hepes pH 6.8 containing 0.1 M sucrose. Diluted fractions were centrifuged at 85 000 x g for 30 min. to pellet membranes. Membrane pellets were resuspended in 1 mL of assay media consisting of 0.05 M Tris-maleate pH 6.0, 0.1 M KCl, and 0.005 M $MgCl_2$. Following incubation for 3 min. at 25 ^{0}C, 1 mL of 8 mM IDP in assay media was added to each suspension. Reaction mixtures were incubated at 25 ^{0}C for 20 min. and stopped with 150 µL of cold 100% trichloroacetic acid.

Inorganic phosphate liberated by the IDPase assay was determined by the method of Ames (1966).

5.2.6.4 Chlorophyll determination (chloroplast marker)

Chlorophyll was determined spectrophotometrically, in 80% acetone extracts, following the method described by Bruinsma (1961).

5.2.7 Electrophoresis, electroblotting, and immunodetection

One dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed as described by Laemmli (1970) with a 10% acrylamide resolving gel using the Bio-Rad Mini-Protean II apparatus.

Proteins were precipitated prior to electrophoresis using a chloroform/methanol/ water system as described by Pohl (1990).

Proteins used for SDS PAGE were dissolved in 1-D PAGE sample buffer containing 60 mM Tris-HCl (pH 6.8), 4% SDS, 0.1 M DTT, 10% 2-mercaptoethanol, 20% glycerol and 0.01% bromophenol blue. All samples were loaded on gels using sterile, disposable gel loader pipet tips (Bio-Rad Laboratories).

Following electrophoresis, gels were either electroblotted to nitrocellulose (Towbin et al. 1979) or stained with coomassie blue.

Nitrocellulose membranes containing electroblotted proteins were blocked overnight in Tris-buffered saline containing 5% (w/v) BLOTTO (non-fat milk powder). Proteins transferred to nitrocellulose membranes were probed with primary polyclonal antibody (1:500) raised in goat against human serum albumin (Sigma Chemical Company). Secondary antibody (1:3000) conjugated to horseradish peroxidase was used to label immunoreactive bands for visualization using enhanced chemiluminescence (Amersham Life Sciences, Oakville, ON, Canada) or antibody (1:5000) conjugated to alkaline phosphatase for visualization using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) in 100 mM Tris buffer pH 9.5 containing 100 mM NaCl, and 5 mM $MgCl_2$. Antibodies were diluted in Tris-buffered saline containing 1% (w/v) BLOTTO (non-fat milk powder) and 0.05% (v/v) Tween 20 (TTBS).

Protein quantity estimation was performed using the bicinchoninic acid (BCA) protein assay (Pierce Chemical).

5.2.8 Tissue printing

Tissue prints on nitrocellulose membranes were prepared by the method of Cassab and Varner (1987). Briefly, nitrocellulose membranes were soaked for 30 minutes in 0.2 M $CaCl_2$ and allowed to dry on filter papers. Fresh petioles were cut into 3 mm thick sections with a razor blade and briefly rinsed with distilled water. Petiole cross sections were placed on the nitrocellulose with forceps and pressed with a gloved fingertip for 15-20 seconds. The tissue section was removed with the forceps and the nitrocellulose tissue print was dried with a stream of air. The nitrocellulose membrane was blocked and treated with antibodies as described previously for electroblots and immunodetection (Section 5.2.7). Control tissue prints were probed with alkaline phosphatase secondary antibodies following the blocking step, and were developed using the alkaline phosphatase colour reaction as described for electroblots and immunodetection (Section 5.2.7). Nitrocellulose prints were rinsed with water, air dried, and photographs were taken using a compound microscope (American Optical, Buffalo, NY).

5.2.9 Chemicals and supplies

Polyclonal human serum albumin antibodies and antibody conjugates were purchased from Sigma Chemical Company. Chemicals used in enzyme assays were of the highest grade available and were purchased from Sigma Chemical Company. Nitrocellulose membrane was obtained from Micron Separations Inc. (Westboro, MA, USA). Chemicals used for alkaline phosphatase colour development were purchased from Fisher Scientific.

5.3 Results

5.3.1 Sub cellular localization

The serum albumin-like plant protein was absent from purified spinach chloroplasts (Fig. 5-1). The serum albumin-like plant protein was present in microsomes but was not detected in the water soluble or cytosolic protein fractions of spinach when cell fractionation and microsomal membrane isolation was performed.

5.3.2 Triton X-114 fractionation of microsomal proteins

Spinach microsomal proteins were partitioned using Triton X-114 and the resulting protein fractions were separated by electrophoresis. SDS PAGE gels were stained with coomassie blue (Fig. 5-2A) or blotted to nitrocellulose and probed with antibodies raised against human serum albumin (HSA) (Fig. 5-2B). HSA antibodies recognized a protein band of approximately 70 kD that was present in the Triton X-114 detergent poor phase, representing peripheral microsomal proteins, but did not react with bovine serum albumin, non-membrane proteins, nor integral microsomal proteins (Fig. 5-2B).

5.3.3 Fractionation of microsomal membranes

Sucrose density fractionation was used to separate the individual classes of membranes, based on enzyme marker activity, from purified spinach microsomes. Figure 5-3A summarizes the measured densities of the sucrose gradient fractions collected and the measured enzyme marker activities for each fraction. Equal amounts of protein from each fraction were loaded on an SDS PAGE gel, western blotted to nitrocellulose for

immunodetection using anti human serum albumin antibodies, and are shown in Fig. 5-3B directly under the corresponding fraction densities and marker enzyme profiles. The immunoreactive spinach protein was concentrated in sucrose gradient fractions of densities ranging from 1.09 to 1.11 gcm^{-3}. The marker enzyme activity peaks for IDPase (Golgi bodies) and NADPH cytochrome c reductase (endoplasmic reticulum) were present in the same fractions as the immunoreactive protein.

5.3.4 Tissue printing

Tissue prints, on nitrocellulose, using leaf petioles of spinach and African violet were incubated with polyclonal anti human serum albumin antibodies and were developed using alkaline phosphatase colour detection. The tissue prints show the presence of the immunoreactive plant protein in the apoplastic space of the petiole cortex tissues of African violet and spinach (Fig. 5-4A). Control tissue prints that were incubated without anti-human serum albumin antibodies showed no alkaline phosphatase reaction (Fig. 5-4B).

5.4 Discussion

To assist in the elucidation of a function for the serum albumin-like protein described in plants, it was necessary to further characterize the protein with respect to its sub-cellular localization.

Chloroplast proteins were examined first for the presence of the serum albumin-like protein because chloroplasts and membrane fragments derived from chloroplasts are a potential constituent of microsomal membrane preparations, when using leaf tissues. Figure 5-1B shows the absence of the serum albumin immunoreactive protein from purified chloroplast preparations, thus allowing the elimination of chloroplasts or chloroplast membrane system fragments as a source of the serum albumin-like protein in the microsomes. Spinach leaf microsomal membrane preparations were used as a source of the serum albumin-like protein and proteins associated with these membranes were partitioned and classified using detergent phase extraction.

The spinach serum albumin-like protein was readily concentrated in the detergent poor phase of the Triton X-114 extraction method which was used for the division of microsomal membranes. This observation indicated that the spinach protein was not tightly bound to or integrated within a lipid bilayer.

Sucrose density gradients and membrane enzyme marker assays have been used effectively to separate and identify different membrane components from microsomal membrane preparations in a number of plant species (Blumwald 1989; Gillott 1980). Separation of different membrane classes from microsomal mixtures is based on the individual densities of specific membrane types and fractionation can be accomplished by passing microsomal membranes through a continuous density gradient. By assaying for

enzymes (markers) which are known to be present only in a desired membrane type, one can determine which fraction of a density gradient contains the targeted membrane.

To determine the classes of microsomal membranes that contained the serum albumin-like spinach protein, leaf microsomal membrane preparations were passed through a continuous sucrose gradient to separate the different classes of membranes according to their densities. Marker enzyme assays were used to confirm the identities of the different membrane fractions within the continuous sucrose gradient. The presence of the serum albumin-like protein was confirmed immunologically in the greatest quantity in fractions corresponding to the endoplasmic reticulum (ER) and Golgi marker enzyme activity peaks (Fig. 5-3).

Proteins that enter the ER-Golgi system can have more than one destination, such as vacuolar deposition or secretion, depending on the presence or absence of specific retention signals (Kermode 1996). No immunological evidence of an ER retention signal (KDEL) sequence was found for the serum albumin-like plant protein when Western blots were probed with anti KDEL antibodies (data not shown), however this evidence is not conclusive because it has been shown that plants often possess the ER retention signal sequence HDEL (Napier et al. 1992) which may not be recognized by anti KDEL antibodies. The observed glycosylation of the serum albumin-like plant protein with galactose residues (Chapter 3) is consistent with the observation that glycosylation with galactose is a common feature of Golgi secreted proteins (De Vries et al. 1988).

The technique of tissue printing onto nitrocellulose membrane is a rapid method by which immunolocalization of proteins can be performed on various plant tissues. Tissue printing is particularly useful for the immunolocalization of loosely associated cell

wall proteins (Cassab 1992). When cut surfaces of plant tissues are pressed against nitrocellulose, which has been soaked with calcium chloride and dried, a printed outline of individual cells and proteins is left bound to the nitrocellulose. Calcium chloride pretreatment of nitrocellulose prior to tissue printing aids the release of proteins (Cassab 1992). Nitrocellulose tissue prints can be probed with antibodies in the same manner as immunoblots, permitting localization of specific proteins.

Using nitrocellulose tissue prints of petiole cross sections, the human serum albumin-like protein was tentatively localized to the cell wall region and intercellular spaces of African violet and spinach.

Many peptides and enzymes are associated with the cell walls of plants and can serve as structural components or may be involved with synthesis or metabolism. Some examples of enzymes reported to be associated with cell walls include: peroxidases, phosphatases, polygalacturonase, pectin methylesterase, and proteases to name a few (Cassab and Varner 1988; Varner and Lin 1989). The serum albumin-like plant protein is likely secreted to the cell wall, however, it is probably not a structural cell wall protein because it was easily dissociated with calcium chloride pre-treatment of the nitrocellulose when tissue printing was performed. It was reported that structural proteins which are immobilized in the cell wall matrix do not transfer to nitrocellulose tissue prints regardless of calcium chloride concentration (del Campillo 1992).

Dhugga et al. (1997) found a peripheral membrane polypeptide that was reversibly glycosylated and which they later localized to the trans-Golgi of *Pisum sativum* L.. They hypothesized that this peptide is involved in xyloglucan or hemicellulose synthesis. With the exception of cellulose, all cell wall polymers are synthesized in the

ER and Golgi apparatus (Gibeaut and Carpita 1994). The mechanism by which these polymers and components get to their destinations within the cell walls is not completely understood, however, the protein described in this dissertation could assist in the delivery of indole acetic acid (IAA), galactose, uronic acids, and (or) phenolic compounds, used for lignin synthesis, to the cell wall.

Fig. 5-1. **Spinach (*Spinacia oleracea* L.) chloroplasts purified using a discontinuous sucrose gradient.** A, Photograph of spinach (*Spinacia oleracea* L.) chloroplasts, purified using discontinuous sucrose gradients, magnified 1000 X. B, Western blot of chloroplast proteins probed with anti human serum albumin polyclonal antibodies. *Lane 1*, human serum albumin (2 μg), *Lanes 2-3*, spinach (*Spinacia oleracea* L.) chloroplast proteins (10 μg). Enhanced chemiluminescent development of Western immunoblotted chloroplast proteins revealed a low molecular weight band with peroxidase activity, clearly visible in lanes 2 and 3.

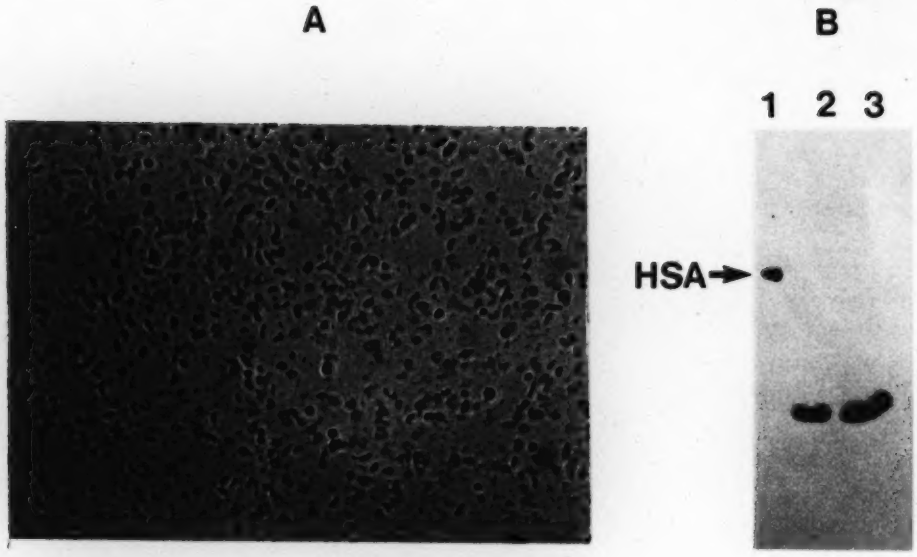

A
B
1 2 3
HSA

Fig. 5-2. **Triton X-114 phase extraction of spinach (*Spinacia oleracea* L.) microsomal membranes.** A, Coomassie blue stained spinach (*Spinacia oleracea* L.) proteins prepared from Triton X-114 fractions. *Lane 1*, human serum albumin (3 μg). *Lane 2*, molecular weight markers (Bio-Rad) as follows: myosin (200 kD), β-galactosidase (116 kD), phosphorylase b (97.5 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), and carbonic anhydrase (31 kD). *Lane 3*, Non-microsomal spinach proteins (60 μg). *Lane 4*, Detergent rich "integral microsomal" phase spinach proteins (60 μg). *Lane 5*, Detergent poor "peripheral microsomal" phase spinach proteins (60 μg). B, Western blot of spinach proteins prepared from Triton X-114 fractions and immunodetection using HSA antisera. *Lane 1*, human serum albumin (3 μg). *Lane 2*, Bio-Rad molecular weight markers. *Lane 3*, Non microsomal spinach proteins (60 μg). *Lane 4*, Detergent rich "integral microsomal" phase spinach proteins (60 μg). *Lane 5*, Detergent poor "peripheral microsomal" phase spinach proteins (60 μg).

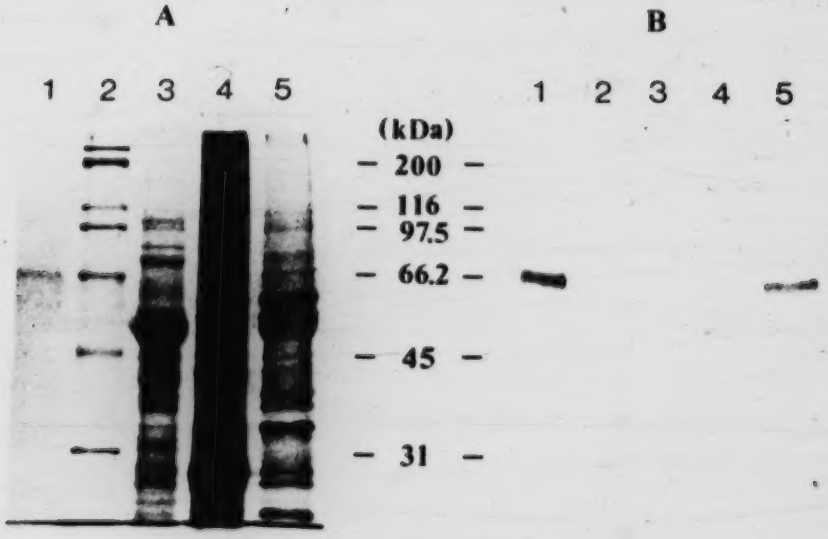
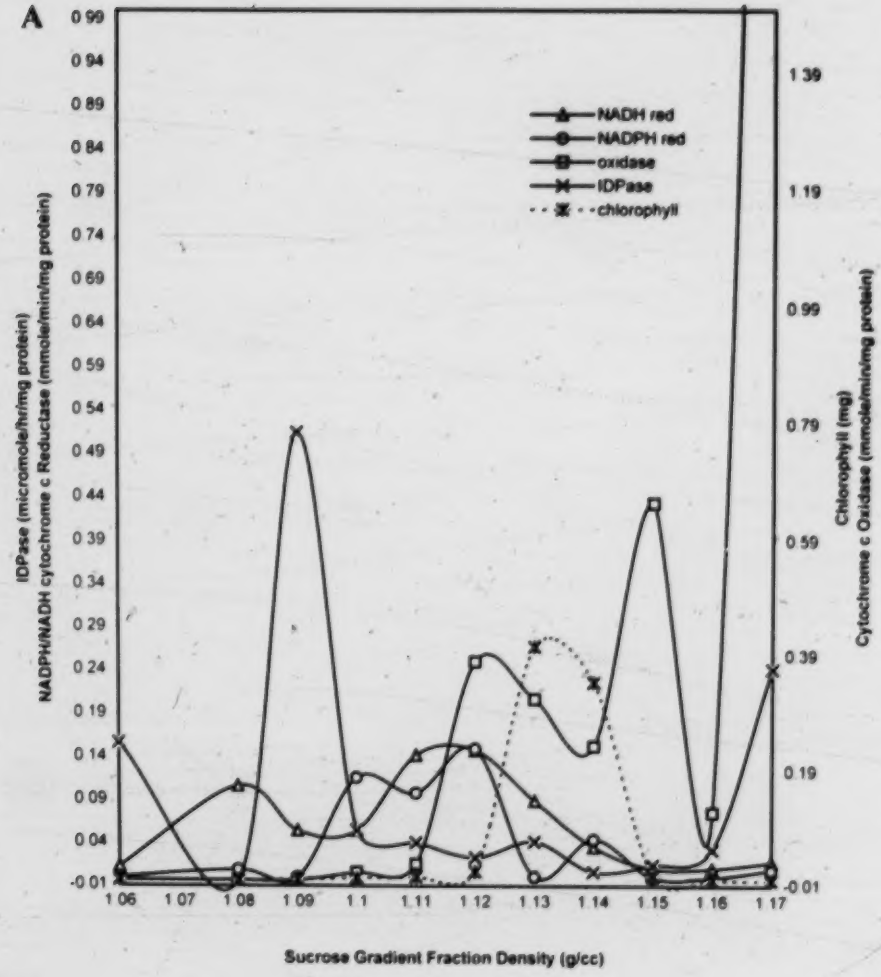

A
B
1 2 3 4 5
1 2 3 4 5
(kDa)
200
116
97.5
66.2
45
31

Fig. 5-3. **Continuous sucrose density gradient fractionation of microsomal membranes from *Spinacia oleracea* L.**. A, Continuous sucrose density gradient fractions from the separation of spinach (*Spinacia oleracea* L.) microsomal membranes and their corresponding membrane marker enzyme activities as follows. NADH cytochrome c reductase (NADH red) = tonoplast, outer mitochondria, and ER membranes; NADPH cytochrome c reductase (NADPH red) = ER; Cytochrome c oxidase (oxidase) = mitochondria; Inosine diphosphatase (IDPase) = Golgi; Chlorophyll (chlorophyll) = chloroplasts.

B, immunoblots of each fraction of the continuous sucrose density gradient (shown in A) probed with anti-human serum albumin polyclonal antibodies. Each lane (gradient fraction) was loaded with an equal quantity (20 μg) of protein.

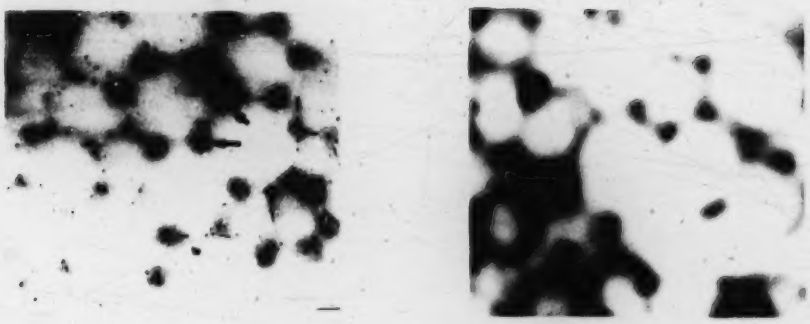
A


NADH red
NADPH red
oxidase
IDPase
chlorophyll
IDPase (micromole/hr/mg protein)
NADPH/NADH cytochrome c Reductase (mmole/min/mg protein)
Chlorophyll (mg)
Cytochrome c Oxidase (mmole/min/mg protein)
0 99
0 94
0 89
0 84
0 79
0 74
0 69
0 64
0 59
0 54
0 49
0 44
0 39
0 34
0 29
0 24
0 19
0 14
0 09
0 04
-0 01
1 39
1 19
0 99
0 79
0 59
0 39
0 19
-0 01
1 06
1 07
1 08
1 09
1 1
1 11
1 12
1 13
1 14
1 15
1 16
1 17
Sucrose Gradient Fraction Density (g/cc)

B

Fig. 5-4. **Tissue prints of plant sections probed with anti human serum albumin antibodies.** A, Nitrocellulose tissue prints of African violet (*Saintpaulia ionantha*) and spinach (*Spinacia oleracea*) leaf petiole cross sections probed with polyclonal anti human serum albumin antibodies and alkaline phosphatase conjugated secondary antibodies following overnight blocking. Arrows show positive staining areas of the blot. B, Control nitrocellulose tissue prints of African violet and spinach leaf petiole cross sections probed only with alkaline phosphatase conjugated secondary antibody following overnight blocking.

A **African violet** **Spinach**



B **African violet** **Spinach**

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Chapter Six

6. PCR amplification of a partial genomic *Spinacia oleracea* L. DNA sharing homology with the NH_2-terminal coding region of the human serum albumin gene.

6.1 Introduction

Human serum albumin is an abundant, multifunctional, plasma protein that serves transport and osmotic regulatory roles in human blood. A protein with NH_2-terminal amino acid sequence homology was identified in plants (Chapter 3) and may represent a functional homolog of the human protein. Some of the ligand binding properties previously described for human serum albumin, which are of potential interest to the study of plants, were shown to occur in the plant homolog. Some of the ligands bound by the plant protein include calcium, indole, and phenolics. In order to determine the extent of the homology between human serum albumin and the plant homolog, described in this thesis, evidence for the presence of a plant gene which codes for the protein was sought.

Human serum albumin is a member of a multi-gene family, arranged in tandem, located in the sub-centromeric region of chromosome 4 (Nishio et al. 1996). However, a gene which codes for a serum albumin-like protein has not been previously reported in plants. The discovery of a hemoglobin gene in non-nodulating plants was the first report of a mammalian serum protein homolog which was present as part of a plant genome (Bogusz et al. 1988). However, there have been reports of other genes in plants which

code for proteins that share striking homology to other human and mammalian proteins (Chapter 2 and references therein).

In this study, polymerase chain reaction (PCR) was used to amplify a genomic DNA fragment from spinach using synthetic oligonucleotide primers (24 and 30 nucleotides long) encompassing the NH_2-terminal amino acid coding region of the human serum albumin gene. Hybridization of a biotin labeled oligonucleotide to Eco RV digested genomic spinach DNA was shown on Southern blots which were probed with alkaline phosphatase labeled anti biotin monoclonal antibodies. DNA sequencing was performed on the PCR products and the partial nucleotide sequences obtained were translated to their corresponding amino acid sequences.

6.2 Materials and Methods

6.2.1 Plant material

Fresh, commercially grown spinach (*Spinacia oleracea L.*) and mature outdoor grown aspen (*Populus tremuloides*) was used for experimental protocols described in this study. Mature leaves were selected for the extraction of DNA that was used in Southern blot hybridizations and polymerase chain reaction (PCR) amplification.

6.2.2 DNA extraction from spinach (*Spinacia oleracea* L.)

Spinach leaves (approximately 0.5 g-1.5 g) were washed in distilled water and blotted dry. Leaves were ground, until finely powdered, in liquid nitrogen. Powdered leaves were added to chloroform-resistant tubes containing 10 mL of CTAB isolation/extraction buffer consisting of 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 25 mM EDTA, 10% CTAB, 0.2% 2-mercaptoethanol, in sterile distilled water. Tubes were mixed well, after leaf powder was added, and were incubated at 60 ^{0}C for 1 hour with gentle shaking every 15 minutes. DNA was extracted with 12 mL of chloroform:octanol 1:1 solution with thorough but gentle mixing by inversion. Tubes were then centrifuged at 9000 x g for 15 minutes to separate the phases. The top aqueous phase was transferred to a sterile test tube and DNA was precipitated by adding chilled isopropanol up to 60% of the total volume. Precipitated DNA was removed using rounded glass hooks and transferred to 5 mL of ethanol-ammonium acetate solution for 30 minutes. DNA was transferred to sterile eppendorf tubes after excess ethanol was squeezed out. DNA pellets were dried and resuspended in TE buffer.

6.2.3 Southern blots

Southern blots were done according to the method described by Sambrook et al. (1989). Restriction digested spinach and aspen DNA was electrophoresed on 1% agarose gels and blotted to positively charged nylon transfer membranes using alkaline conditions to denature the DNA during the Southern transfer. When transfer of DNA was complete, blots were baked at 80 ^{0}C for 2 hours prior to hybridization analysis.

6.2.4 Oligonucleotide hybridization

Oligonucleotide hybridization was performed on Southern blotted spinach total genomic DNA which was restriction digested with the Bam HI and Eco RV. Southern blots, which had been previously baked at 80 ^{0}C for 2 hours, were incubated in a solution consisting of 2x SSC, 0.1% SDS, and 50 μg/mL proteinase K for 1 hour at 37 ^{0}C with gentle shaking. Blots were rinsed twice with 2x SSC for 5 min. at room temperature. Blots were placed in glass hybridization bottles with a blocking solution consisting of 4% BLOTTO (5% non-fat milk powder, 0.02% sodium azide), 6x SSC, 0.05% sodium pyrophosphate and incubated at hybridization temperature for 15 minutes before each biotin labeled oligonucleotide probe (described in section 6.2.7 below) was added. Hybridization temperature for oligonucleotide #1 was 56 ^{0}C and for oligonucleotide #2 was 53 ^{0}C. Biotin labeled oligonucleotide probe (1 μL of 20 μM oligonucleotide per 20 mL of blocking solution) was added to each hybridization bottle and incubation was continued for 2 hours. Blots were washed twice in 2x SSC, 0.05% pyrophosphate at room

temperature for 5 minutes. A final wash was performed in 2x SSC for 30 minutes at a temperature 12 ^{0}C higher than the hybridization temperature. Southern blots were then stored in Tris-buffered saline until biotin label detection could be performed.

6.2.5 Biotin label detection

Biotin labels on oligonucleotide probes, which were hybridized to restriction digested DNA on Southern blots, were detected using commercial (Sigma Chemical Company) alkaline phosphatase conjugated anti-biotin monoclonal antibodies (diluted 1:5000 in Tris-buffered saline containing 1% (w/v) non-fat milk powder and 0.05% Tween 20 (TTBS)). Colour development of bands with bound antibody was performed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) in 100 mM Tris buffer pH 9.5 containing 100 mM NaCl, 5 mM $MgCl_2$

6.2.6 PCR conditions

Polymerase chain reaction (PCR) amplification of spinach genomic DNA (1 ng) was performed using a Perkin-Elmer 9600 PCR system, programmed for 25 cycles with the following protocol: denaturation at 94 ^{0}C for 30 sec., annealing at 50 ^{0}C for 60 sec. , extension at 68 ^{0}C for 60 sec., with a final extension at 72 ^{0}C for 2 min.

6.2.7 PCR primers and biotin labeled oligonucleotides

Two human serum albumin oligonucleotide primers were designed to represent regions within the human serum albumin gene as follows:

Primer #1 5'-GAT GCA CAC AAG AGT GAG GTT GCT CAT CGG-3' which corresponds to amino acids D A H K S E V A H R located from amino acid residues 1-10 at the NH_2-terminus of human serum albumin.

Primer #2 5'-CTG TTT GGC AGA CGA AGC CTT CCC-3' which corresponds to amino acids G K A S S A K Q located from amino acid residues 189-196 within human serum albumin.

Oligonucleotides used for Southern blot hybridizations were synthesized exactly as above with a biotin label attached at the 5' end of each primer DNA sequence. Primer #1 = Oligonucleotide #1

Primer #2 = Oligonucleotide #2

6.2.8 DNA sequencing

PCR products were sequenced using the dideoxy chain termination method described by Sanger et al. (1977) with fluorescent dye terminators. Automated DNA sequencing of the PCR products was performed on an ABI 370A DNA sequencer (Applied Biosystems, Foster City, California) using Applied Biosystems protocols. Oligonucleotides #1 and #2 (described in section 6.2.7) were used as primers for automated sequencing reactions. DNA and deduced protein sequences of the PCR products were compared with National Center for Biotechnology Information (USA) databases using BLAST software (Altschul et al. 1990) and translated amino acid sequences were obtained using the ExPASy translate tool (Appel et al. 1994).

6.2.9 Chemicals and supplies

Biotin labeled (5') oligonucleotide probes and PCR primers were synthesized by National Biosciences Inc., (Plymouth, MN, USA). Taq-DNA polymerase used for PCR amplification and nylon transfer membranes used for southern hybridizations were purchased from Boehringer Mannheim. DNTP's, $MgCl_2$, and PCR buffer were purchased from Perkin-Elmer. Alkaline phosphatase conjugated anti-biotin monoclonal antibodies and other chemical reagents used in this study were purchased from Sigma Chemical Company.

6.3 Results

6.3.1 Southern blot hybridization

Only synthetic oligonucleotide probe #1, designed to be complementary to NH_2-terminal coding region of the human serum albumin gene, hybridized with purified spinach genomic DNA when it was restriction digested with Eco RV (Fig. 6-1).

6.3.2 PCR amplification

Polymerase chain reaction of spinach genomic DNA with primers which were complementary to the NH_2-terminal coding region of the human serum albumin gene resulted in the amplification of several PCR products (Fig. 6-2).

6.3.3 DNA sequence analysis

PCR products were sequenced using the chain termination method (Sanger et al. 1977). Each PCR product gave a similar truncated DNA sequence (Fig. 6-3A). Each time sequencing of the PCR products was attempted, a strong nucleotide signal was produced but suddenly dropped to the baseline (Appendix II).

Several PCR product sequences were translated to their corresponding peptides and gave a similar result in the 3'5' reading frame (Fig. 6-3B). The translated peptides were aligned with human serum albumin and show similarity to the NH_2-terminal amino acid sequence of the human protein (Fig. 6-3B). BLAST database searches using the PCR product nucleotide sequences failed to reveal any similarity to known DNA sequences.

6.4 Discussion

Immunological evidence for the presence of a plant protein with homology to human serum albumin was presented in Chapter 3. To support the theory of an endogenous serum albumin-like protein in plants, a search for genetic evidence was initiated using several molecular biology protocols.

Attempts to isolate a cDNA clone from the expression of phage vectored cDNA libraries (λZAP) were unsuccessful, possibly due to a low abundance of the target mRNA during the library construction or the loss of rare clones during library amplification. Other factors reducing the possibility of success during screening of the expression libraries with antibodies is based on the limitations of the antibodies themselves. Expressed proteins lack post-translational modifications which might be necessary for an antibody to recognize an antigenic determinant. In addition, the reading frame in which clones were inserted into the expression vector would need to be correct in order to get the correct peptide, and even then, it would need to be an epitope that could be recognized by the antibody.

In order to test the feasibility of performing a PCR amplification of plant genomic DNA which codes for the serum albumin-like plant peptide sequence, Southern blotted plant DNA was probed with synthetic oligonucleotides which code for previously described NH_2-terminal binding domains of human serum albumin. The biotin labeled synthetic oligonucleotide probe (#1), which was complementary to the first 10 amino acids of human serum albumin, hybridized to Eco RV digested total spinach DNA.

Southern hybridization suggests the presence of a gene which codes for the NH_2-terminal amino acid sequence described in Chapter 3.

Problems were encountered when nucleotide sequencing of PCR products was attempted and was likely due to the formation of tertiary DNA structures. A truncated nucleotide sequence was obtained for each PCR product before the sequencing signal dropped and when the nucleotide sequences were translated to peptides, in the 3'5' direction, similarity to the NH_2-terminal amino acids of human serum albumin was observed (Fig. 6-3B).

BLAST analysis of the truncated nucleotide sequences of the PCR product failed to show any similarities to known nucleotide sequences. Differences between the nucleotide sequence of the amplified plant DNA which codes for the serum albumin-like protein and the human gene which codes for the NH_2-terminal amino acids of HSA might exist due to the degenerate nature of the genetic code or differences in codon usage frequency between plants and animals. Degenerate oligonucleotide primers designed with codon usage frequencies specific to spinach might work better to amplify the serum albumin-like target DNA sequence.

The nucleotide sequence which codes for the NH_2-terminal amino acid region of HSA does not have any reported plant homologues despite the observation of a conserved plant protein homolog which shares similar properties with HSA. The nucleotide sequencing results of this study are preliminary and cannot discount the possibility that a plant gene with homology to human serum albumin is present in spinach.

Fig. 6-1. **Southern blot hybridization of a synthetic human serum albumin oligonucleotide probe to spinach (*Spinacia oleracea* L.) genomic DNA.**

A, Electrophoresis of restriction digested aspen (*Populus tremuloides*) and spinach (*Spinacia oleracea* L.) genomic DNA. *Lane λ*, digested Lambda, *Lane 1* Total spinach DNA Bam H1, *Lane 2* Total aspen DNA Bam HI, *Lane 3* Total spinach DNA Eco RV, *Lane 4* Total aspen DNA Eco RV. B, Southern blot, of agarose gel shown in A, hybridized with biotin labeled olgonucleotide probe #1 (5'-GAT GCA CAC AAG AGT GAG GTT GCT CAT CGG-3'). Hybridization was detected (shown by arrow) using alkaline phosphatase conjugated anti biotin antibody and nitro blue tetrazolium colour development.

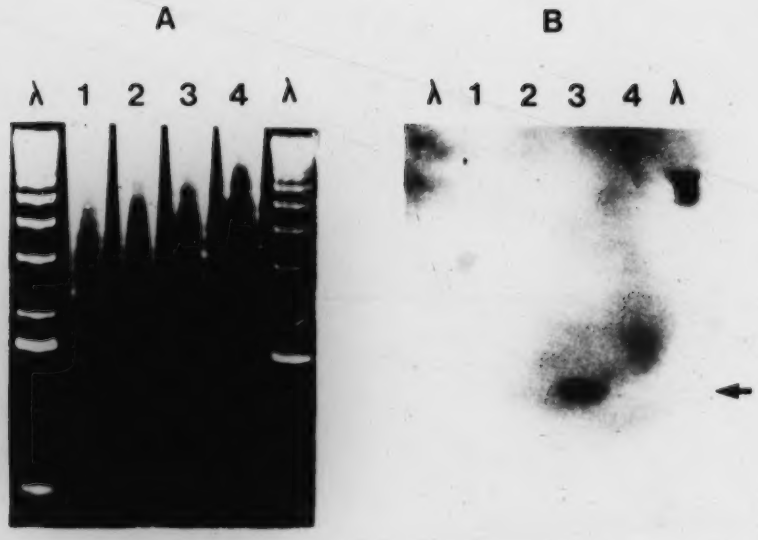
A
λ 1 2 3 4 λ
B
λ 1 2 3 4 λ

Fig. 6-2. **PCR amplification of a partial genomic serum albumin-like clone using spinach (*Spinacia oleracea* L.) DNA and synthetic oligonucleotide primers for human serum albumin.** *Lane 1* Low DNA mass Ladder (Gibco). *Lane 2* PCR products.

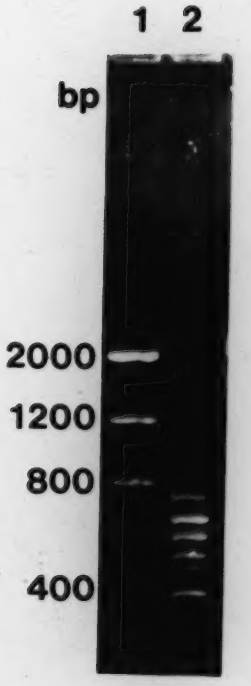
1
2
bp
2000
1200
800
400

Fig. 6-3. **Nucleotide sequence of a partial genomic PCR product from spinach (*Spinacia oleracea* L.) DNA, amplified using primers which were complementary to human serum albumin.**

A, Partial nucleotide sequences of PCR products obtained using spinach (*Spinacia oleracea* L.) DNA. B, Amino acid sequence translation of the PCR products with the partial nucleotide sequences shown in A. Translated amino acid sequence regions common to all PCR products (1-4) and showing similarity to the NH_2-terminal amino acid sequence of human serum albumin (5) are underlined.

A

1)CNCNNTTTCTCTNCCCACGNACCGAATNGTGTGCATCANAGNNTTGGGCAA
TCANNNNNNTNNNNNGAATNNCCTTTTTCTNNTCATNCGTC

2)GGGCTTTNCGANTGAGGGCCTNACACTTGTGTGCATCACTGTTTGGCANCA
CNGAAGNCTTCCCNANTAGGGACCACAATCTTNTCTNCATCANGGT

3)TCTNTNTNGCCTCTTCTTNTNTGCATCACTGTTTGGCATCACTATNNCTTCC
CCTTTTTGGCCTCTCTCTTTTCTCCTTNNCTGTNTGN

4)CCGAGGNANAACCTNCACNCTTNGNTTTGCATCACTGTTTGGGTANANNAA
AANGNTCGAAGATTNGGGAAATGAAAGGAGAGNNCNCCNNGNTTGGTGCG
TTAG

B

1) 3'5' translation
TXXXKKXIXXXXXIAQXXDAHXSXRGXRXX

2) 3'5' translation
X**Met**XXRLWSLXGKXXVXPNSDAHKCXALXXKA

3) 3'5' translation
XQXRRKERGQKGEXIV**Met**PNSDAXKKRXXR

4) 3'5' translation
LTHQXXXXLLSFPXSSXXXXYPNSDAXXXVXPR

5) Mature Human serum albumin
DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQ

6.5 References

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7. Synthesis and conclusions.

7.1 Synthesis

This dissertation describes a protein found in plants which shares significant NH_2-terminal amino acid sequence homology with human serum albumin. The amino acid sequence data obtained for the spinach protein described in this thesis matches 15 residues of the NH_2-terminal amino acid sequence identified in human serum albumin as being responsible for binding of calcium and indole derivatives (McMenamy 1977; Geisow and Beaven 1977), two important plant growth regulating chemicals. I was able to show calcium and indole binding by the purified spinach (*Spinacia oleracea*) protein. The nine NH_2-terminal amino acids of the HSA sequence were described as being required for the formation of a "pocket" structure which is required for the binding of indole derivatives (Geisow and Beaven 1977).

A survey of recent literature on the topic of amino acid sequence homologies between proteins found in plants and animals revealed many reports of conserved regions within amino acid sequences which are found in both plant proteins and animal homologs (Chapter 2). Striking homologies between amino acid sequences have been noted between plant and human proteins. Cases of amino acid sequence identity which exist between plant and animal proteins have been described as being conserved for the purpose of maintaining peptide structure or function at key active sites (Shafqat et al. 1996; Rafferty et al. 1996; Caffrey et al. 1991). The plant protein described in this dissertation is likely another example of this conserved structure or function relationship between a particular amino acid sequence found within proteins of disparate organisms. While there are striking similarities

between the serum albumin-like plant protein and human serum albumin, some very important differences in their chemistry indicate that the two proteins are not entirely alike. A comparison between human serum albumin and the plant protein is summarised in Table 1. Glycosylation (Brennan et al. 1990) and the lack of immunoreactivity with monoclonal antibodies raised against human serum albumin are very important properties distinguishing the plant protein as being different from human serum albumin.

Table 1 Comparison of human serum albumin with the plant serum albumin-like protein

Characteristic	Human serum albumin	Plant serum albumin-like protein
(NH_2) amino acid sequence	DAHKSEVAHRFKDLG	DAHKSEVAHRFKDLG
Antibody reactivity	anti-HSA polyclonal anti-HSA monoclonal	anti-HSA polyclonal anti-Calreticulin *
Calcium binding	Yes**	Yes
Indole binding	Yes**	Yes
Glycosylation	No (rarely yes)	Yes
Molecular mass	66.2 kD	70 kD (est)
Isoelectric point	4.7-5.5	6.4-6.6

* cross reactivity

** theoretical characteristic but not observed for experimental controls

Human serum albumin is a multifunctional protein with some very specific binding properties, yet the major function hypothesised by researchers is the relatively simple role of maintaining osmotic pressure in the blood (Brown and Schockley 1982). Human serum albumin has a number of specific binding properties, which are determined by the NH_2-terminal amino acid sequence of the protein and would theoretically serve multiple, useful, functions in plant cells. Human serum albumin has been shown to bind IAA (Bertuzzi et al. 1997). The binding of polyphenolic compounds and tannins by HSA has been reported (Tsarevskii and Karal'nik 1975) and the serum albumin-like plant protein appears to have similar properties (Chapter 4).

Final thoughts...

In light of the results included in this dissertation and similar discoveries of other plant proteins with striking homology to human and other mammalian proteins, there are some general philosophical questions to consider in the context of previously documented protein discoveries.

In a situation where a hypothetical protein is found first in humans, does this necessarily preclude all other organisms, including plants, from sharing amino acid sequence identity? Suppose that the same hypothetical protein was discovered first in plants and then later discovered in humans, would there be equal difficulty proposing why it was found in humans and how it got there?

The rare glycosylated form of serum albumin reported in humans (Brennan et al. 1990) might suggest a possible evolutionary link between the plant protein described here and human serum albumin since the plant protein is glycosylated and the common form of

human serum albumin in present day humans is not. Evolutionary arguments for the parallel transfer of the human serum albumin gene from humans to plants are tempting but difficult to prove. However, many authors of reports describing homologous proteins found in both plants and animals suggest a common ancestral origin of amino acid sequences prior to the divergence of plant and animal kingdoms (Ferl et al. 1994; Lopez et al. 1994; Gupta 1995). The vast majority of protein research has been done in animal systems, and many proteins have been studied in detail long before plant homologues were ever considered. As a result of technological advances in protein research, it is likely that many previously unreported plant proteins with striking homology to human proteins will be discovered in the future.

7.2 Conclusions

Using calreticulin antibodies which exhibit cross reactivity to human serum albumin, a protein with NH_2-terminal amino acid sequence homology to human serum albumin was discovered in plants. This previously unreported plant protein was detected immunologically in several different plant species using commercially available polyclonal antibodies raised against human serum albumin (Chapter 3).

The immunoreactive protein was purified to near homogeneity (Chapters 3 and 4) and was partially characterized chemically. NH_2-terminal amino acid sequence was obtained for the protein from peas and spinach. Striking amino acid sequence homology with human serum albumin was observed at the NH_2-terminus for the protein purified from both plant species (Chapter 3).

While there are striking similarities between the amino acid sequences of this plant protein and human serum albumin, some physical and chemical differences are apparent. Chemical staining with the thymol/sulfuric acid method as described by Racusen (1979) indicated that carbohydrates were associated with the protein and GC-MS analysis showed the presence of galactose and galacturonic acid in association with the serum albumin-like plant protein (Chapter 3). The association of sugars or uronic acids with the plant protein, which are not observed with human serum albumin, might explain observed differences in electrophoretic mobility and chemical staining between the plant protein and commercially prepared human serum albumin. Human serum albumin has binding sites for calcium and indole derivatives (McMenamy 1977; Geisow and Beaven 1977). When purified by HPLC, the serum albumin-like plant protein was shown to bind both calcium and the indole derivative 5-[^{3}H]N_3IAA (Chapter 3).

Continuous sucrose density gradients, polyclonal antibodies raised against human serum albumin, and membrane enzyme marker assays were used to localize the protein to the ER-Golgi secretory system of spinach (Chapter 5). Nitrocellulose prints of plant tissue cross sections were probed with anti human serum albumin antibodies and suggest that the serum albumin-like plant protein might accumulate in the apoplast or the cell walls following its movement through the ER-Golgi secretory system.

Southern blots of total spinach DNA cleaved with Eco RV were probed with a synthetic oligonucleotide probe which represents the nucleotide sequence responsible for coding the NH_2-terminal amino acids of human serum albumin. A biotin labelled oligonucleotide probe for the NH_2-terminal coding region of human serum albumin recognized a spinach derived Eco RV genomic DNA fragment on a Southern blot (Chapter 6).

Polymerase chain reaction (PCR) was performed on spinach genomic DNA using oligonucleotide probes designed against two different sites encompassing the NH_2-terminal amino acid coding region of the human serum albumin gene. A set of PCR products was obtained and sequenced to provide evidence for the existence of a plant gene(s) which codes for an amino acid sequence homologous to the NH_2-terminus of human serum albumin. PCR amplification of the target sequence from spinach required highly purified template DNA and produced several products of varying size. This suggests the possibility that the target sequence might be the result of a gene with multiple alleles, or part of a multi-gene family.

Based on the observed chemical properties and subcellular localization of the serum albumin-like plant protein, one can speculate with respect to its possible functions. The

plant protein is secreted via the ER-Golgi secretory pathway to the cell walls and has galactose and uronic acids, which are both components of pectin (Bacic et al. 1988), associated with it. The presence of the serum albumin-like protein in both young and mature plant tissues, and the possible presence of phenolic lignin precursors associated with the protein (Chapter 4) also supports the suggested involvement of the protein in cell wall synthesis. Many components of the cell wall are synthesized or processed in the Golgi bodies prior to secretion. However, few proteins involved with the transport of these components have been characterized. It is possible that the serum albumin-like plant protein is involved with the transport and deposition of substances required for cell wall synthesis, while calcium and indole binding by the plant serum albumin-like protein suggest that Ca^{2+} and IAA might influence the activity of the protein described in this study.

7.3 Recommended future studies

Further research is required to enhance the understanding of the protein described in this thesis. Cloning of the entire gene that codes for the serum albumin-like plant protein might be attempted using a set of degenerate oligonucleotide PCR primers. The degenerate primers should represent the nucleotides which code for the NH_2-terminal amino acid sequence of the plant protein. The resulting PCR product could be labelled and used as a probe to screen DNA libraries for more complete cDNA clones of the plant serum albumin-like protein gene. The translated cDNA sequence data will provide information about possible structural similarities between human serum albumin and the plant protein, and could reveal other potential ligand binding domains.

The importance of further protein study should not be overlooked. Protein function is best studied with, and in some cases requires, enzymes that retain their native conformations. The study of various ligand binding properties of the serum albumin-like plant protein might require purified, non-denatured forms of the protein which would only be possible after further refinement of the purification methods described in this dissertation. Further amino acid sequence data, including internal peptide sequencing, would provide more structural information and would provide additional target sequences for which degenerate sets of PCR primers could be designed.

A survey of serum albumin-like protein sequences from various plant species would allow comparison of the inter-relatedness of evolutionarily distinct plants and might allow one to trace the origin of the serum albumin-like protein. With this information, researchers might determine approximately when, or if, parallel gene transfer occurred between plants and humans.

7.4 References

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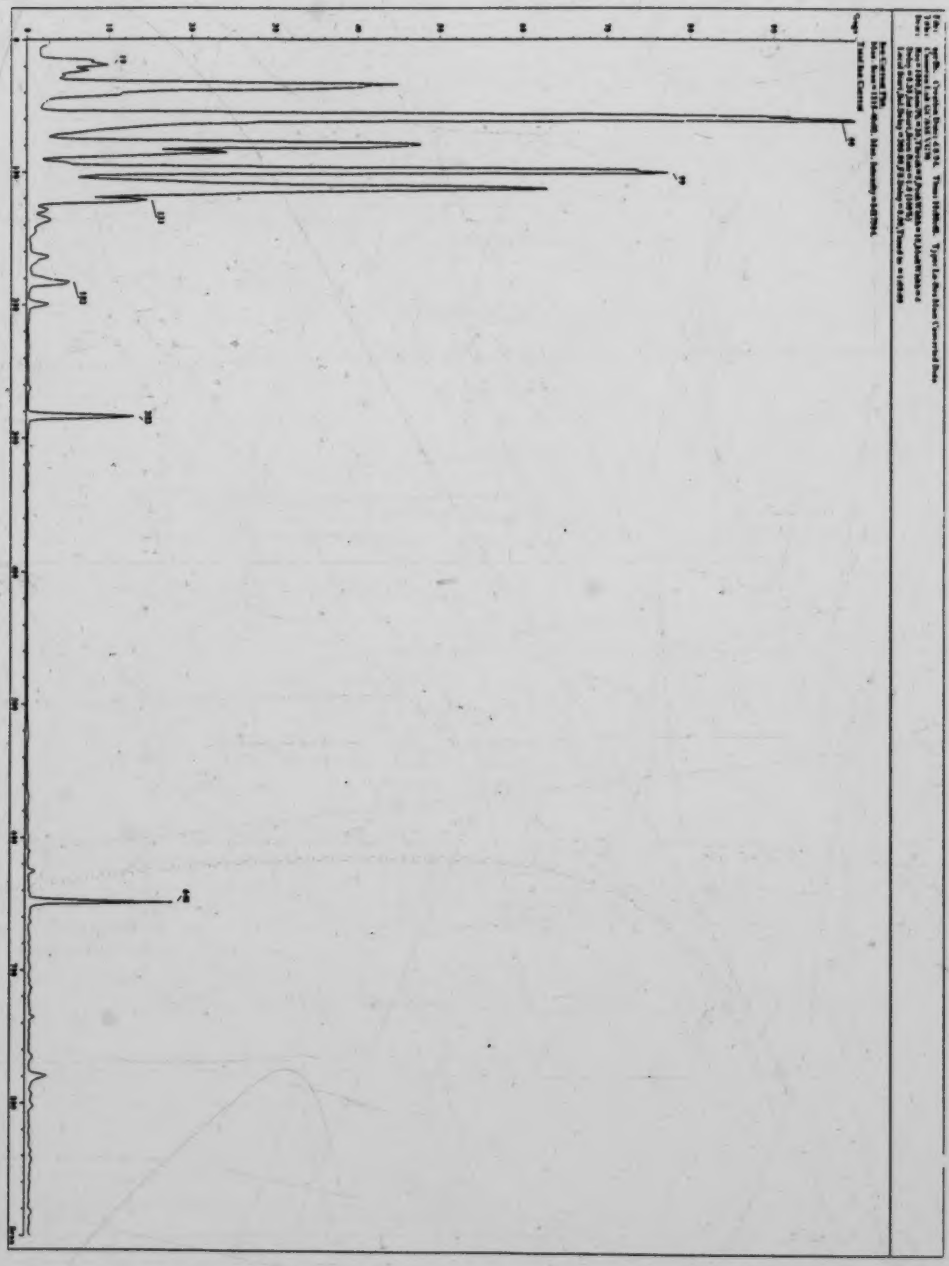
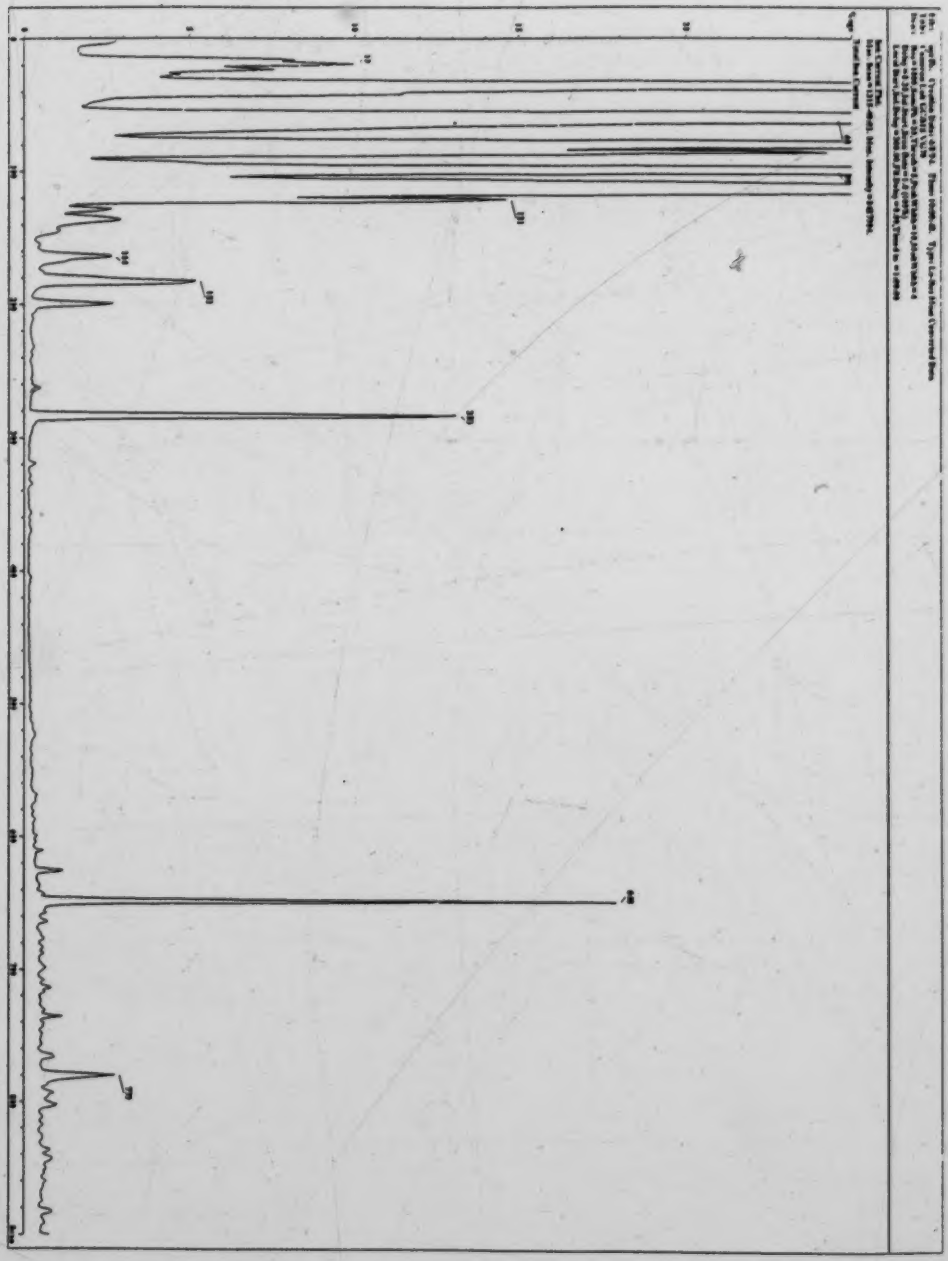
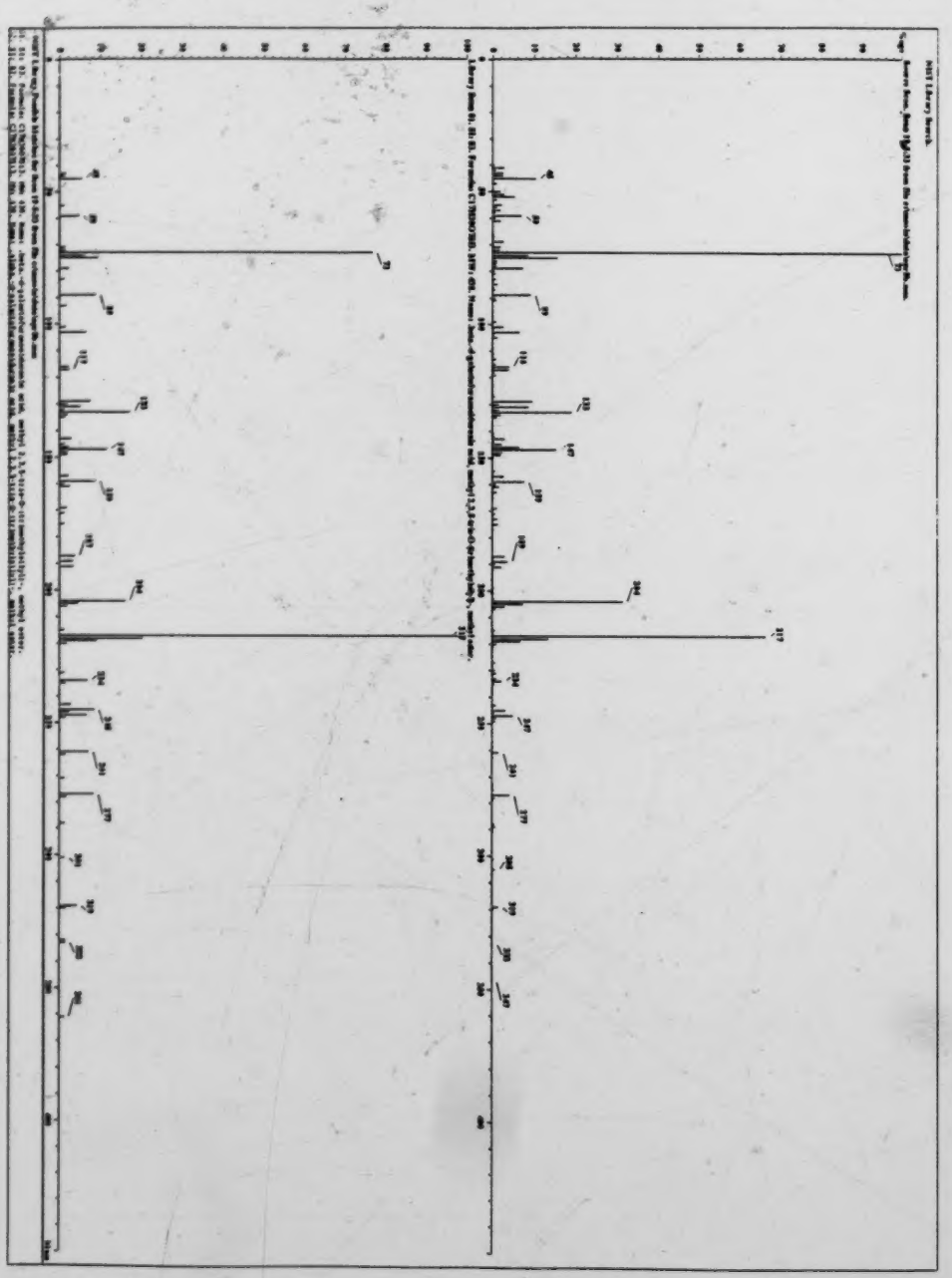
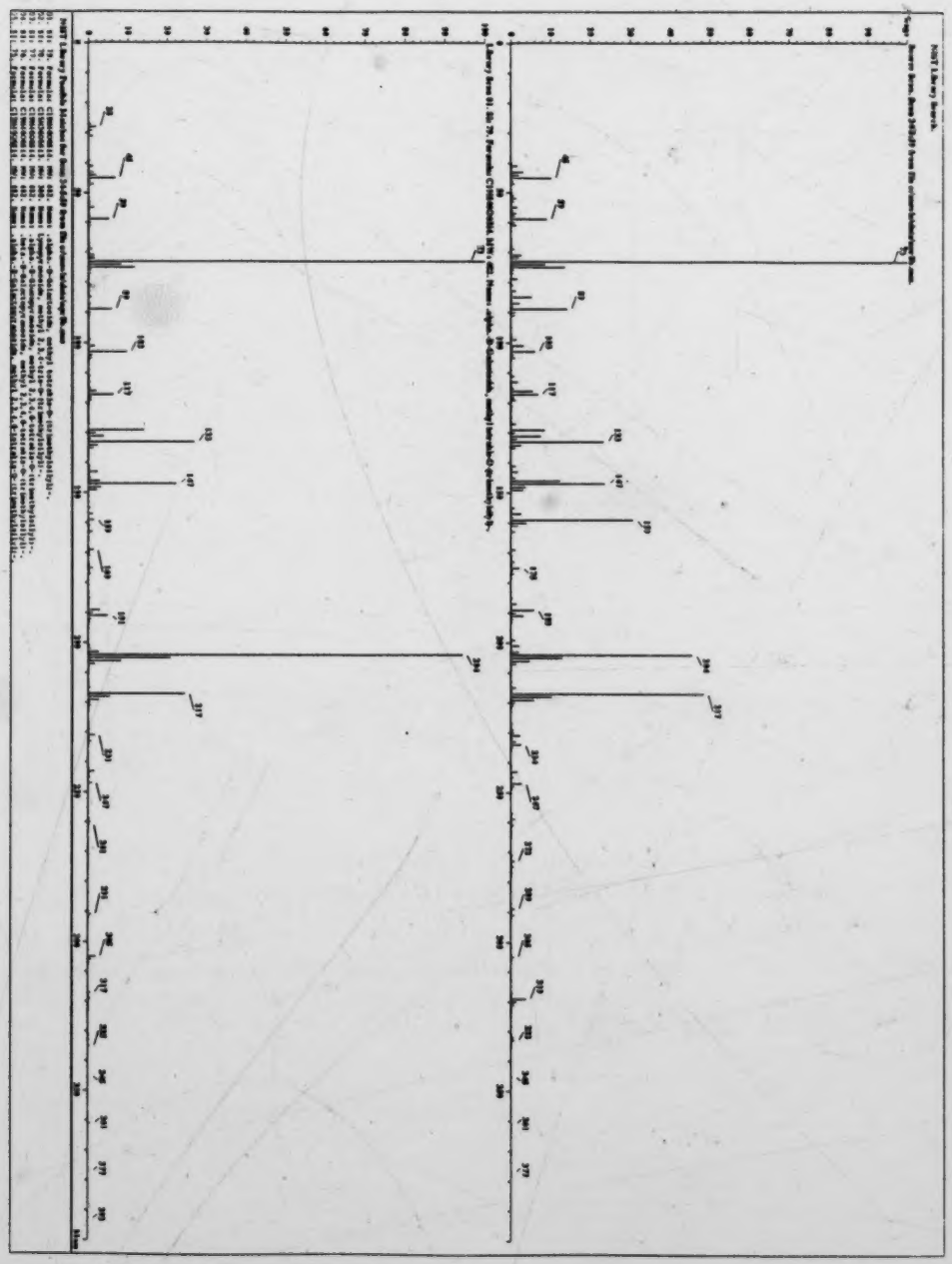
Appendix I

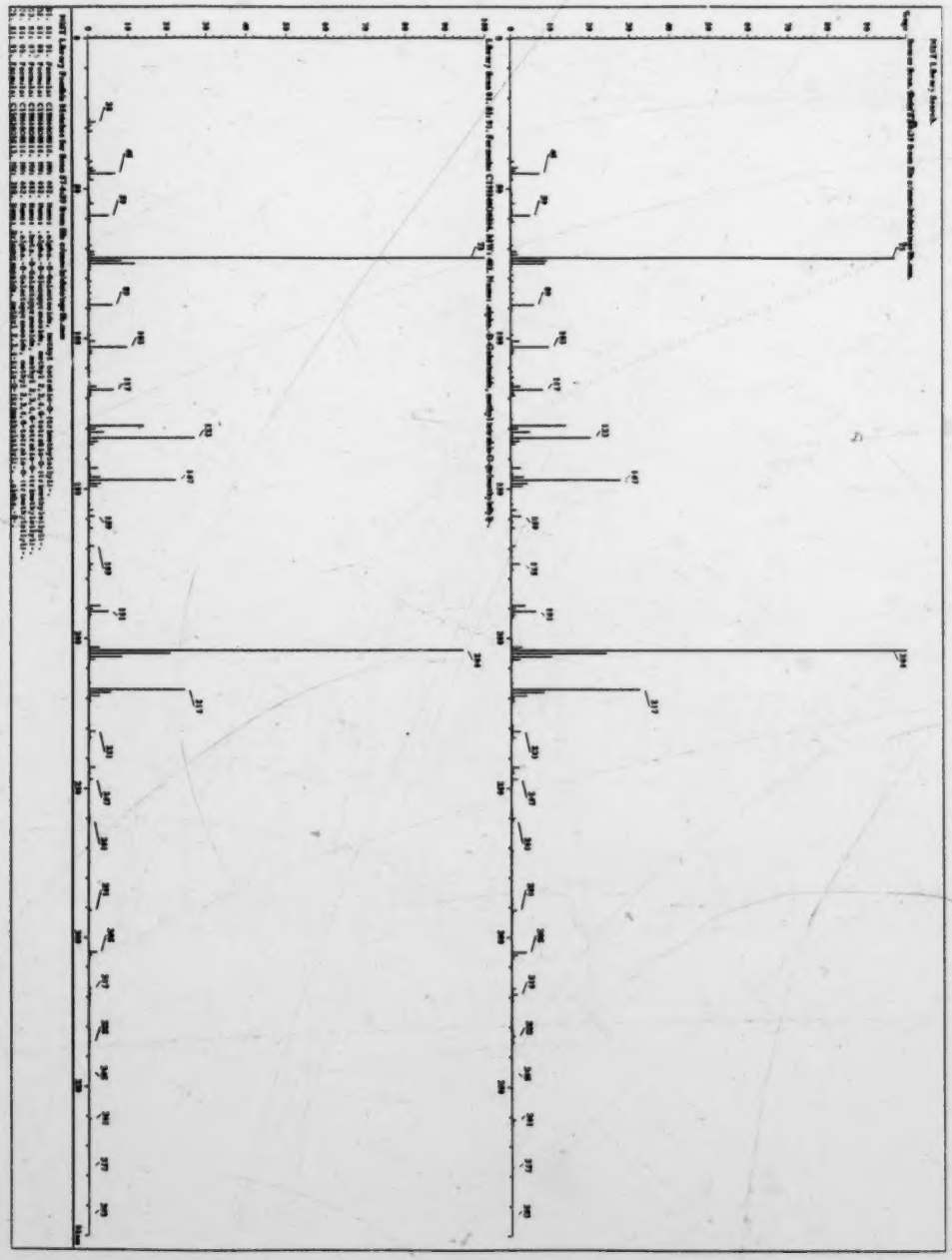
Gas chromatogram and mass spectra for sugars associated with spinach (*Spinacia oleracea* L.) serum albumin-like protein.

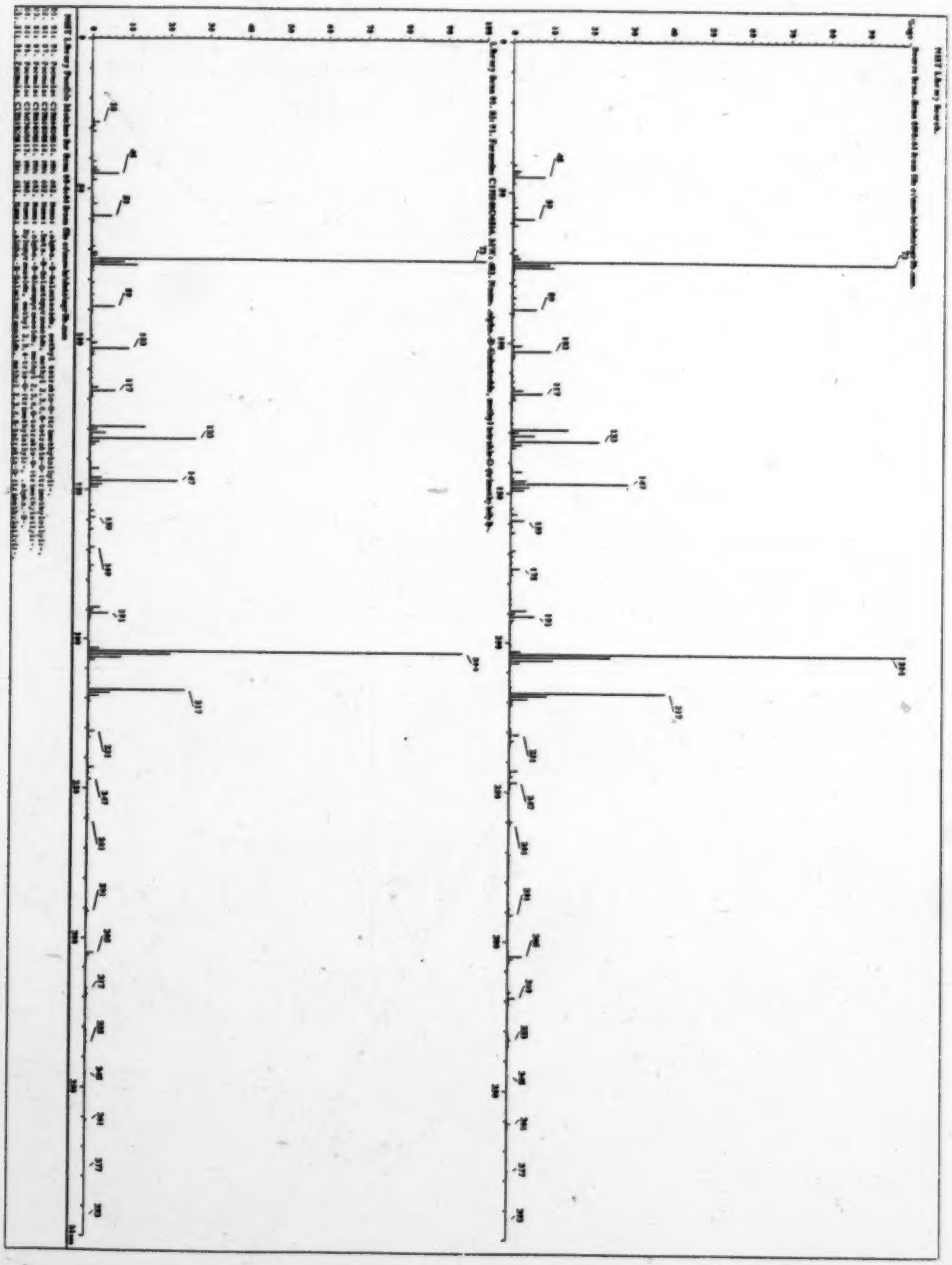


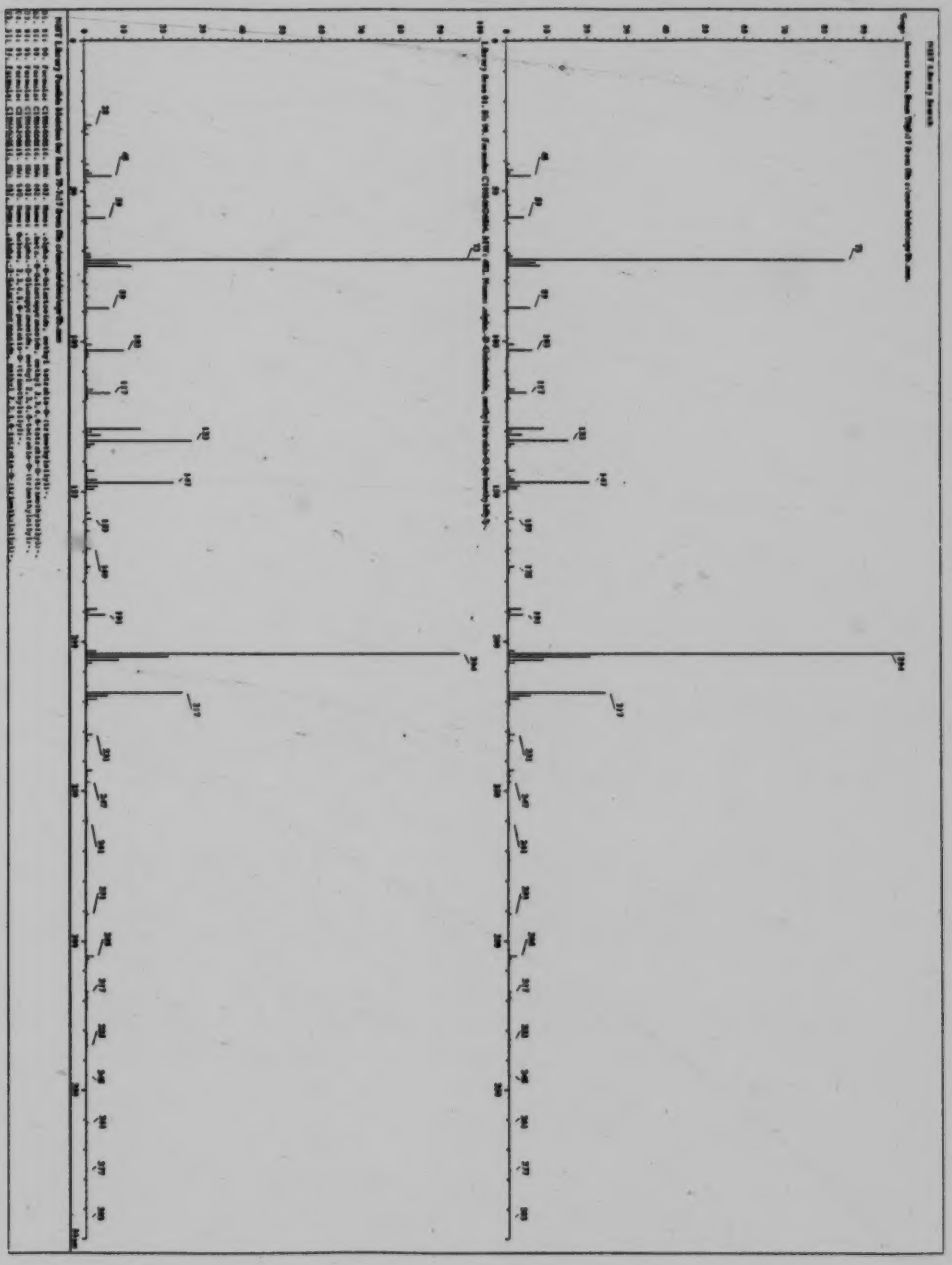


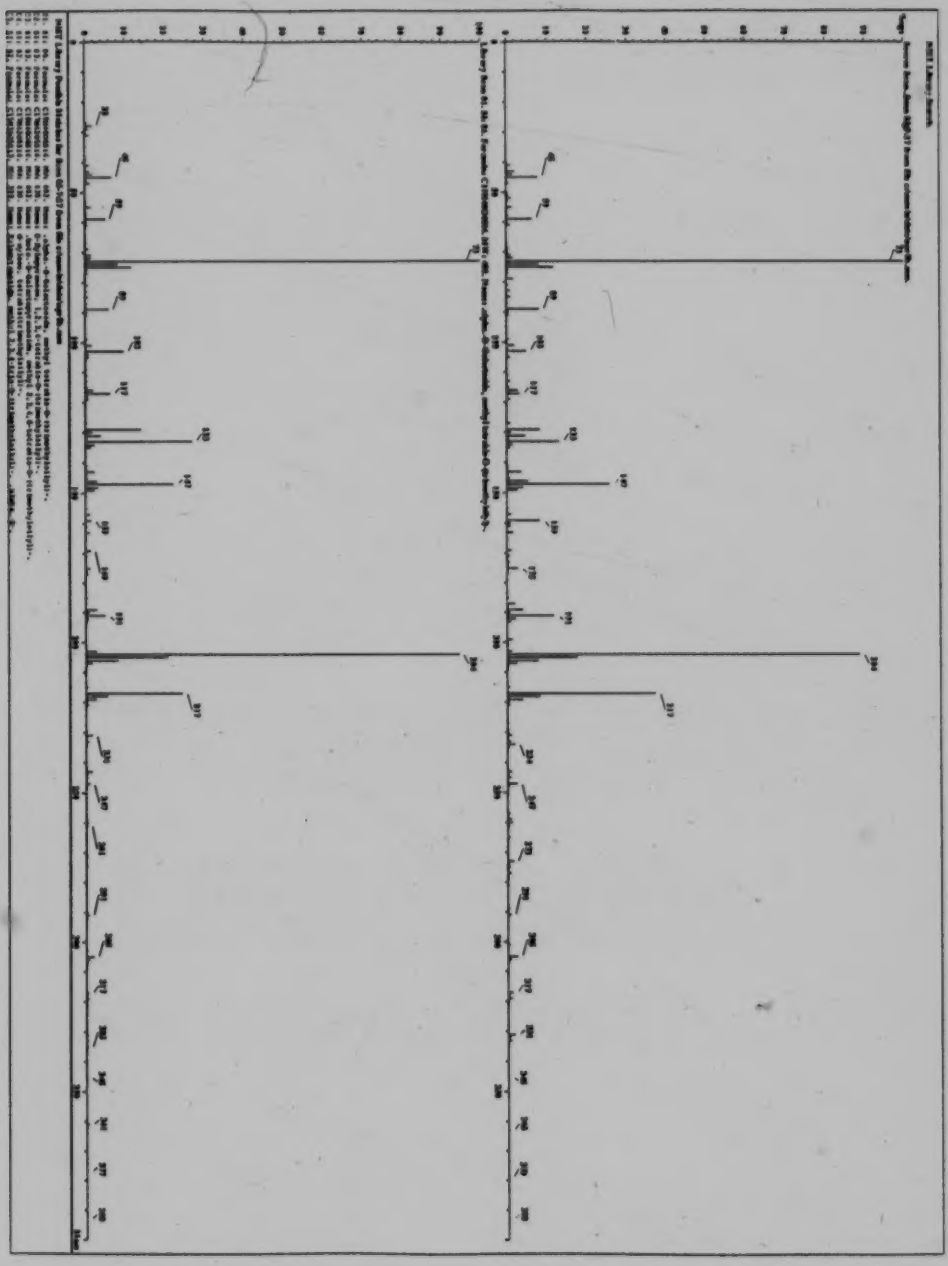


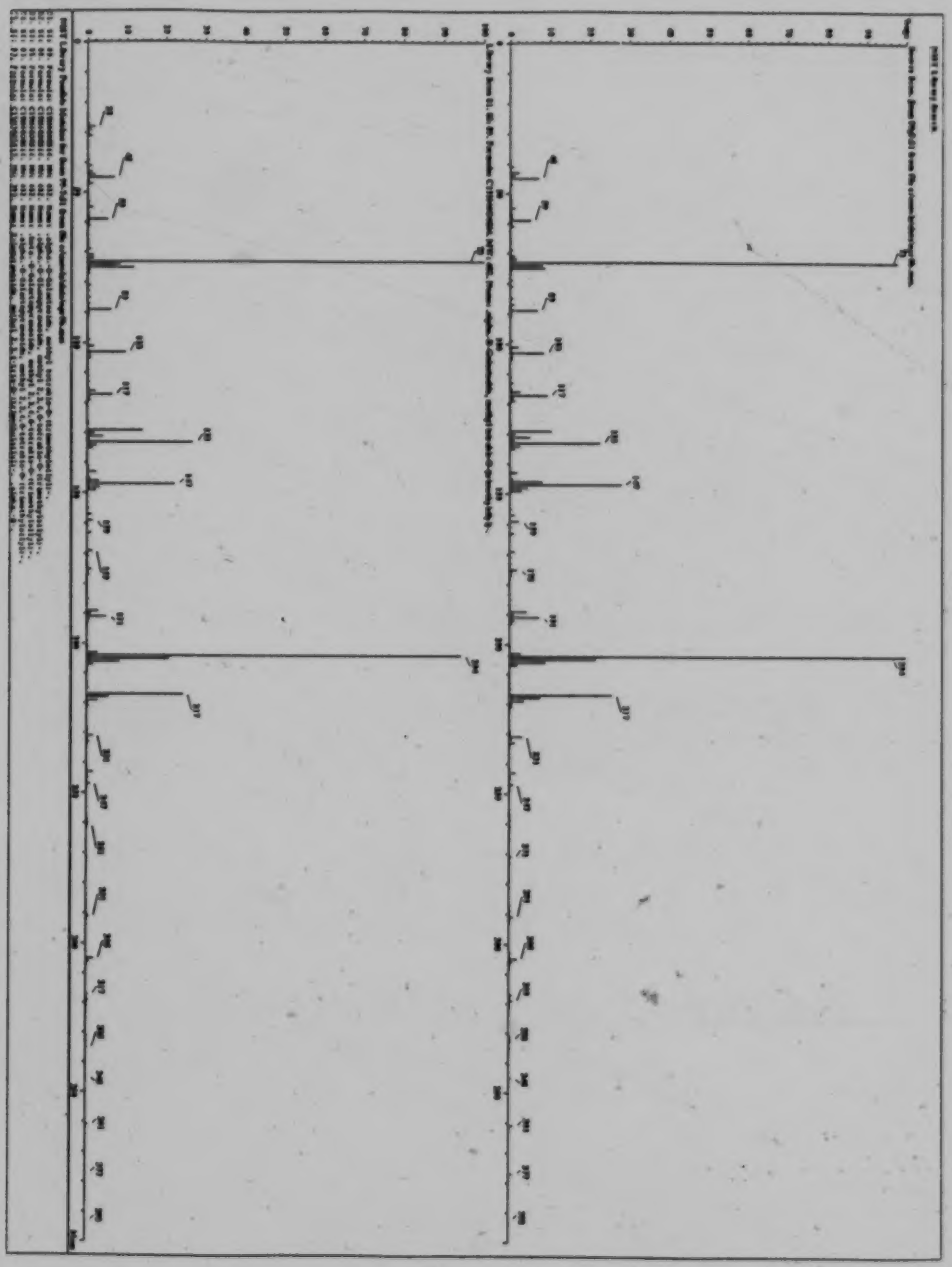


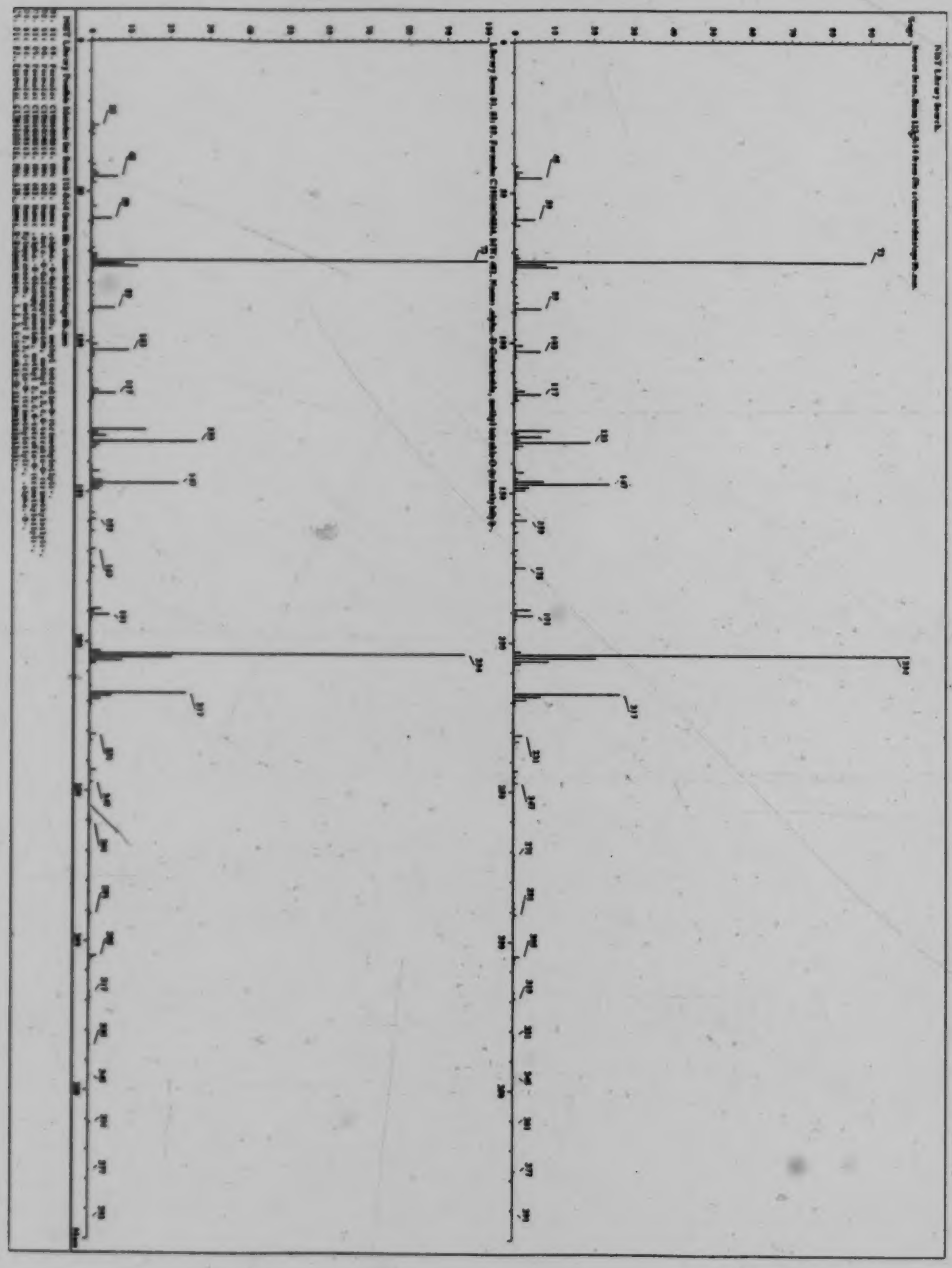


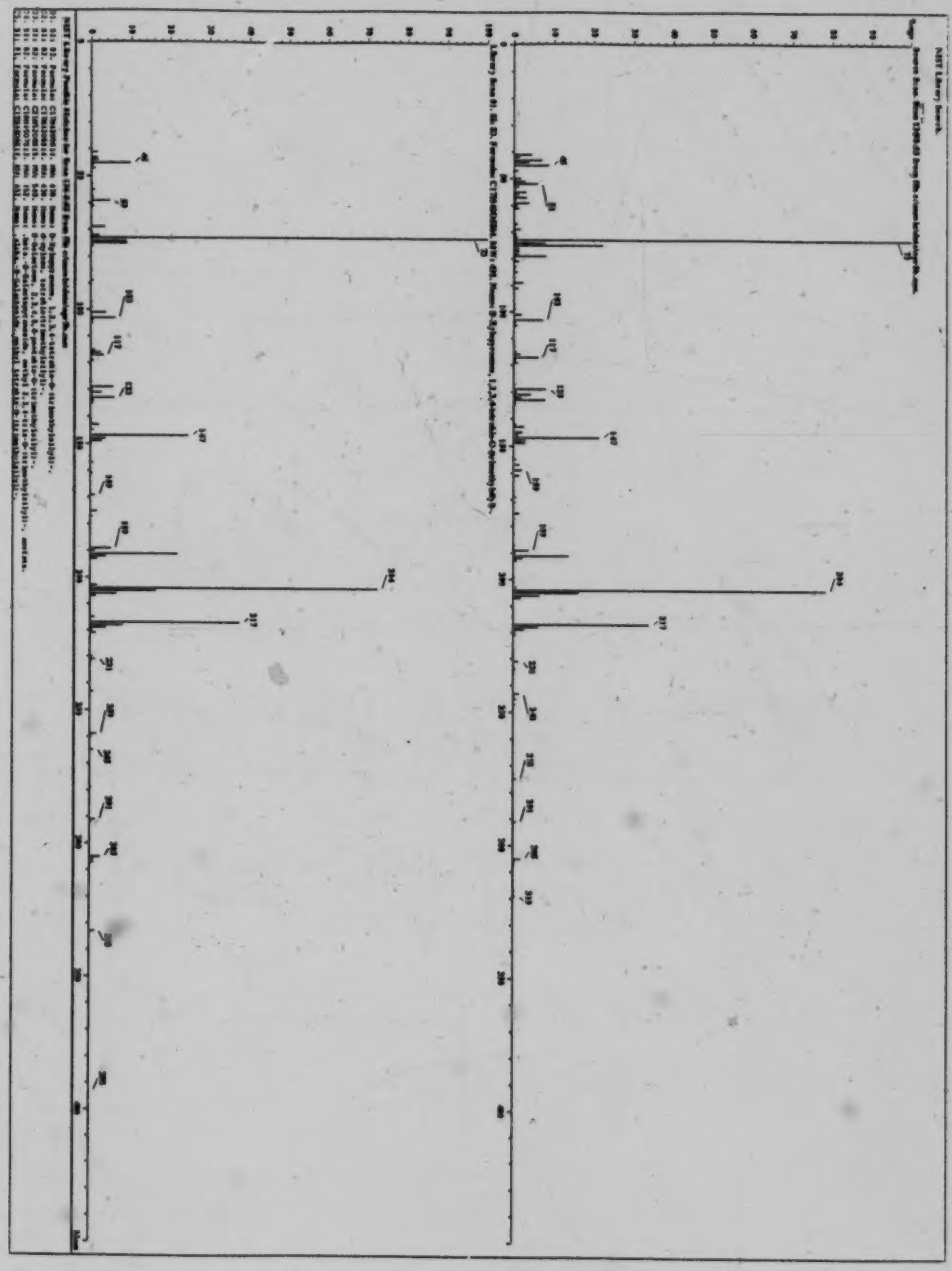


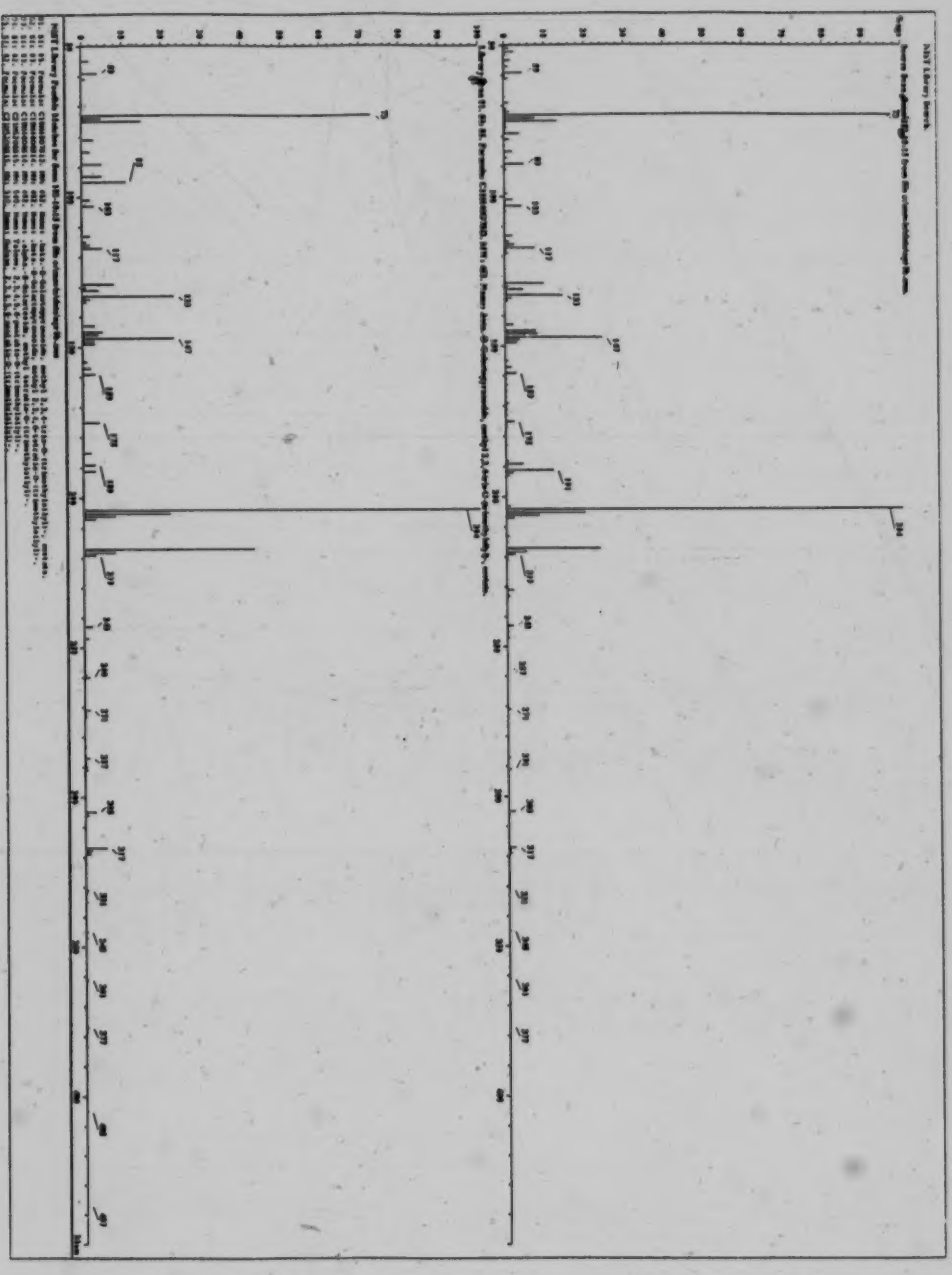


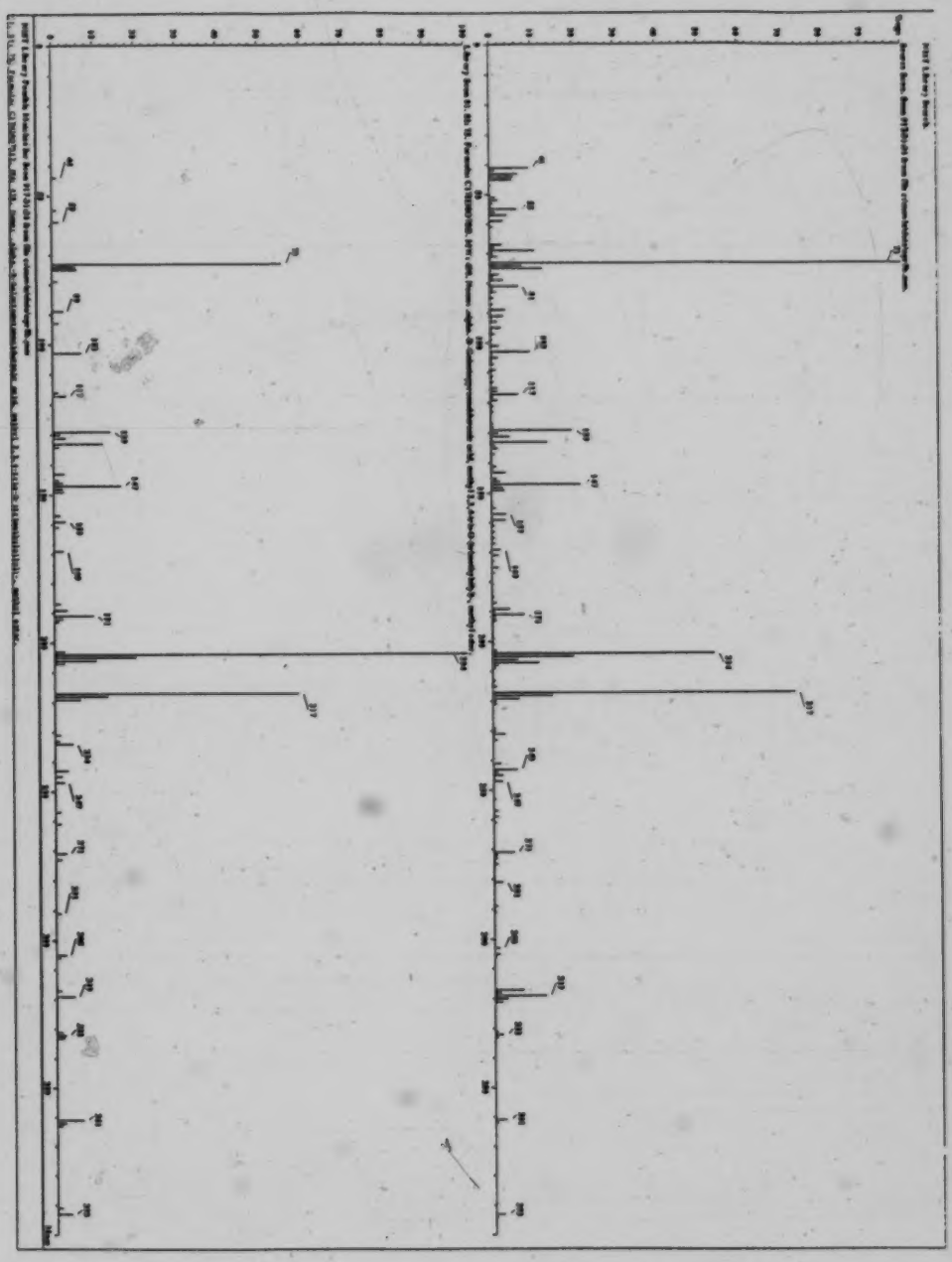


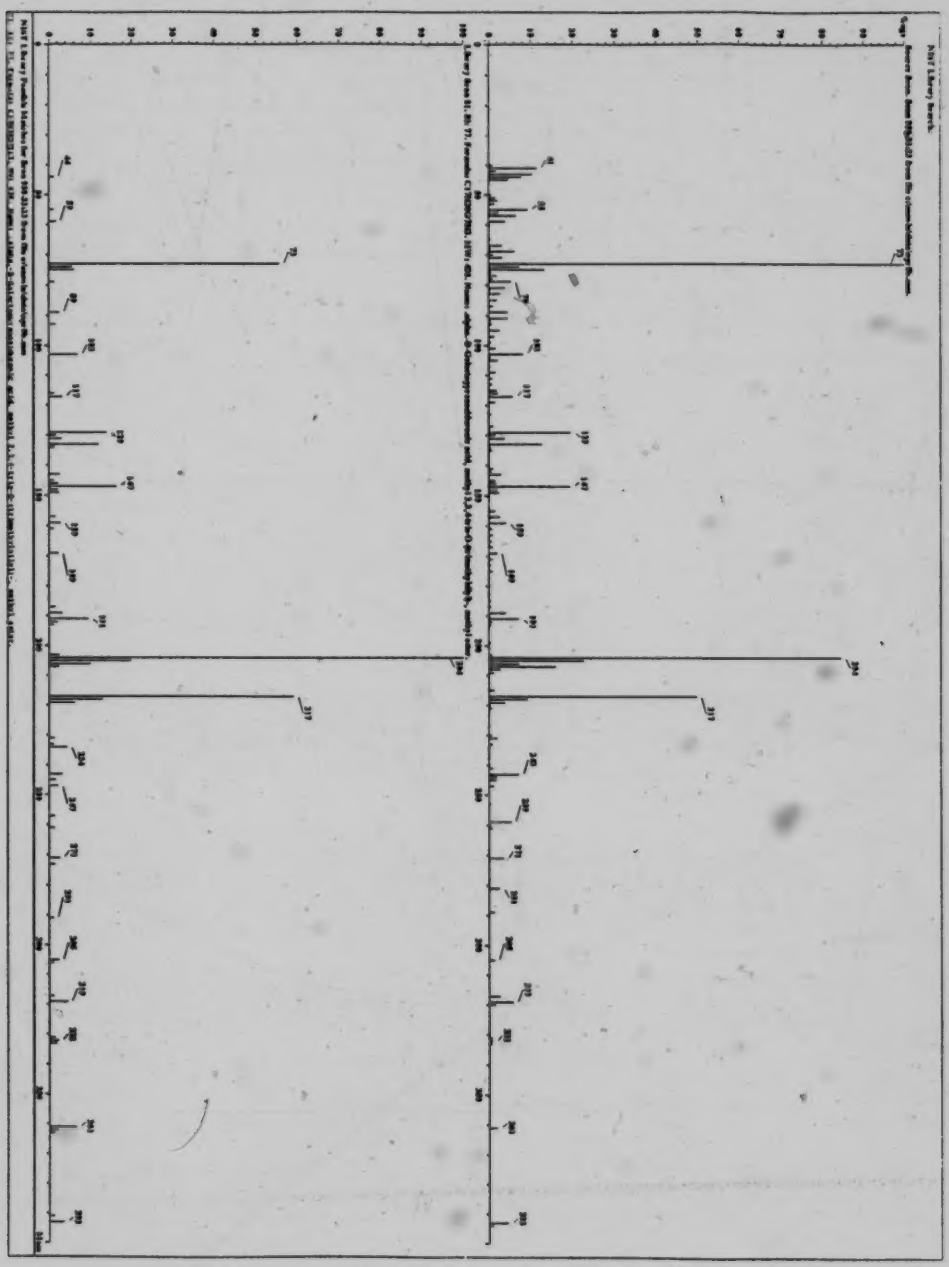






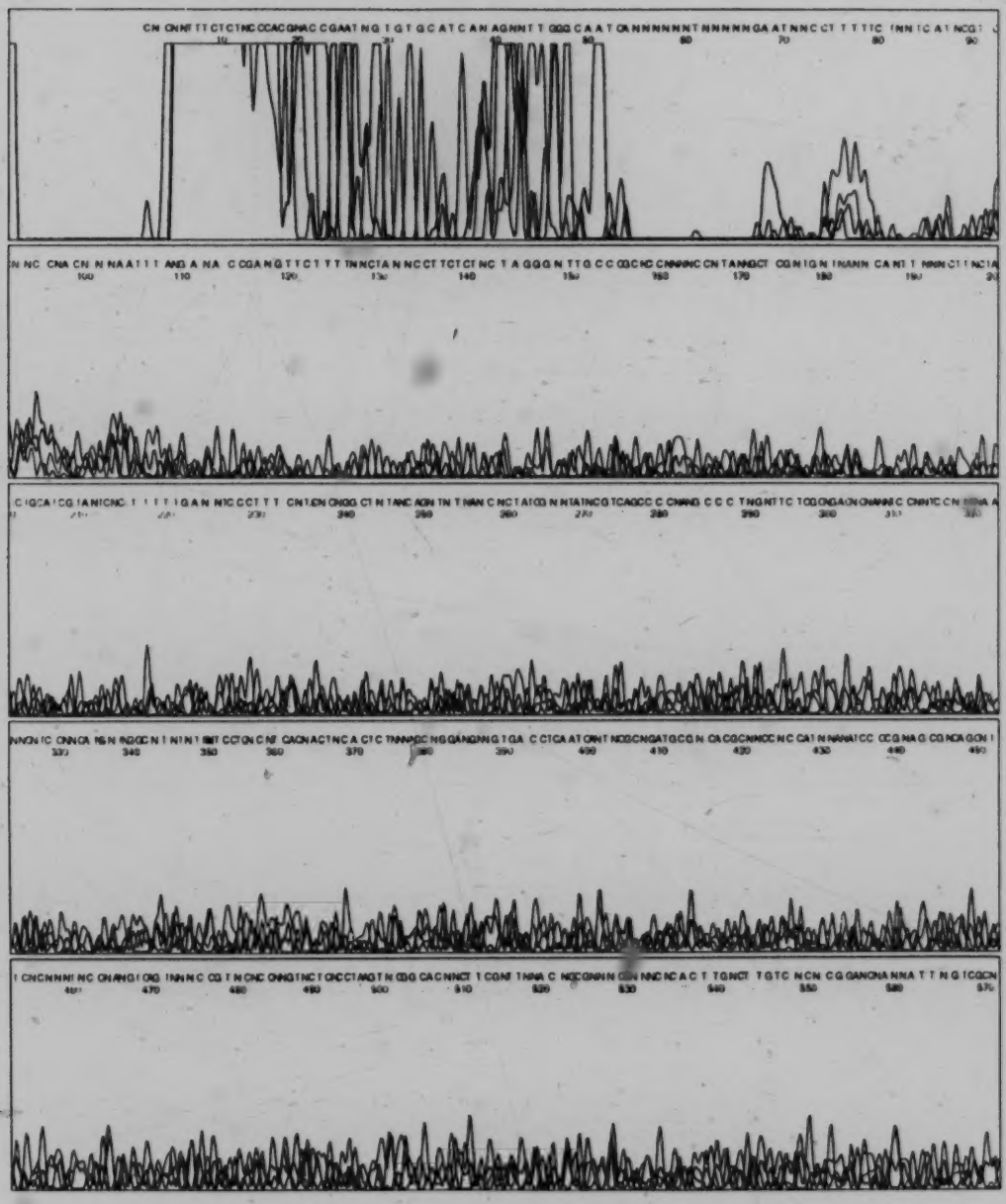


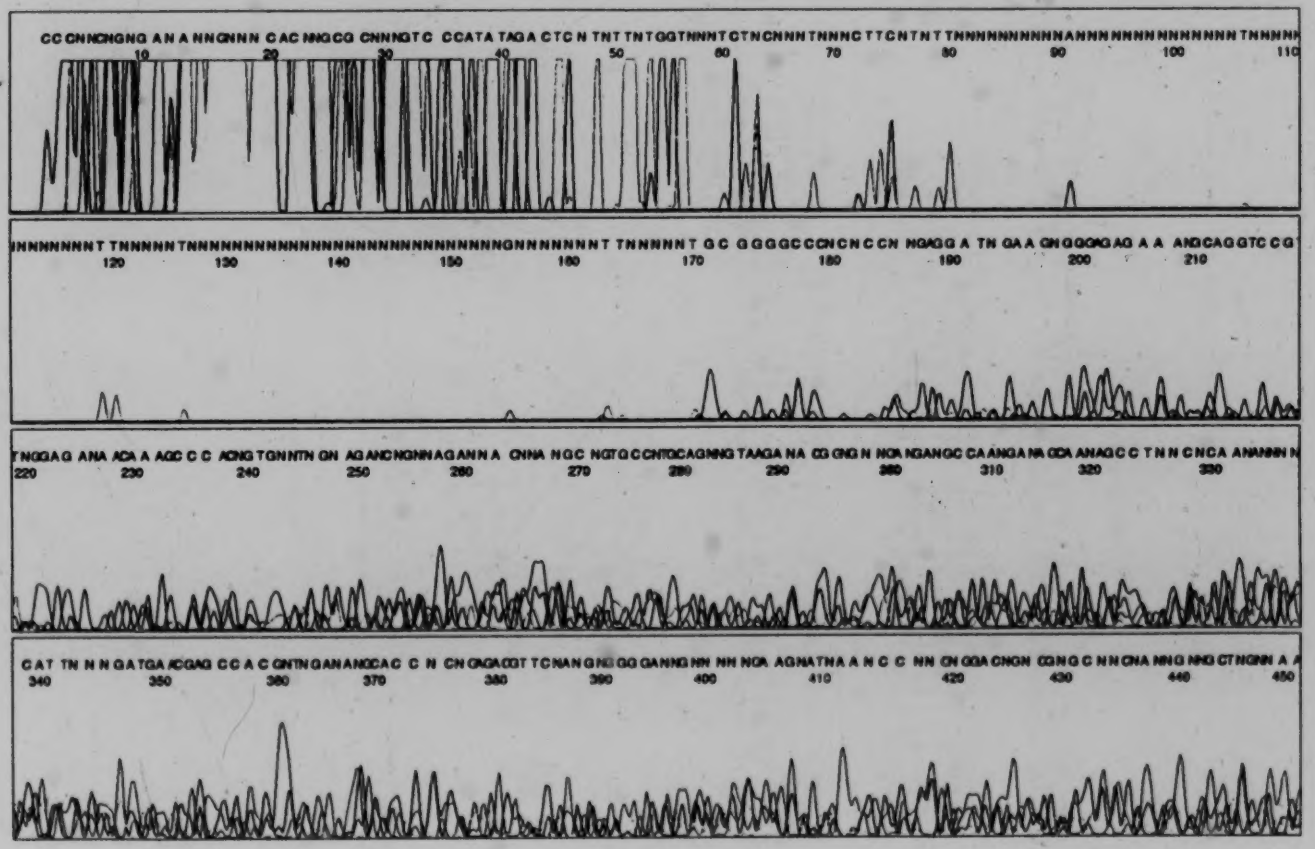


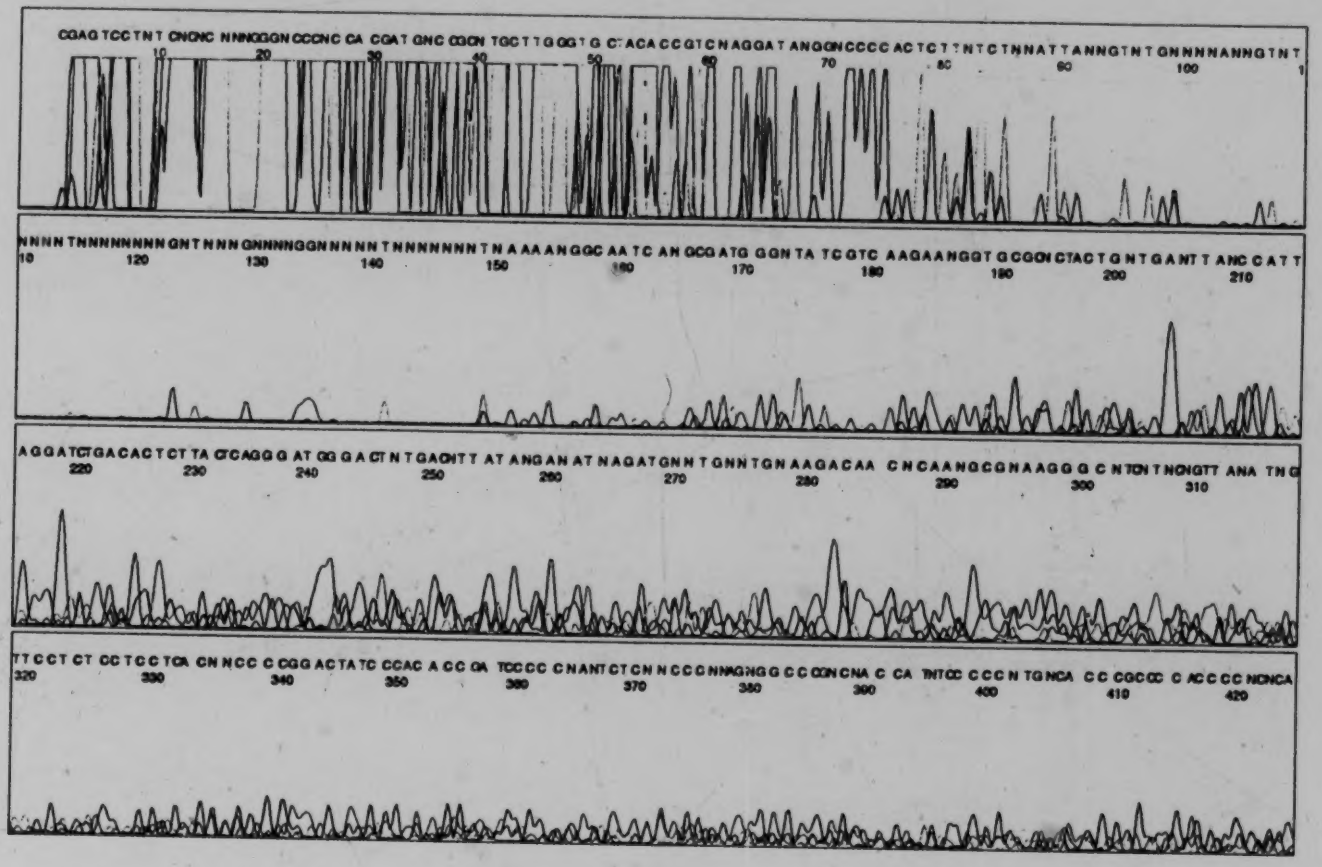


Appendix II

Automated nucleotide sequence analysis of PCR products obtained from spinach (*Spinacia oleracea* L.) DNA.







Appendix III

List of abbreviations

μM - Micromolar
1-D PAGE - One dimensional polyacrylamide gel electrophoresis
2-D PAGE - Two dimensional polyacrylamide gel electrophoresis
5-[^{3}H]N_3IAA - Tritiated 5-azidoindole-3-acetic acid
BCA - Bicinchoninic acid
BCIP - 5-Bromo-4-chloro-3-indolyl phosphate
BLAST - Basic local alignment search tool
BSA - Bovine serum albumin
DEAE - Diethylaminoethyl
DNTP - Deoxy nucleotide triphosphate
DTT - Dithiothreitol
EDTA - Ethylenediaminetetraacetic acid
EGTA - Ethylene glycol-bis(β-aminoethyl ether)-NNN',N'-tetraacetic acid
ER - Endoplasmic reticulum
fw - Fresh weight
GC-MS - Gas chromatography - Mass spectrometry
HPLC - High performance liquid chromatography
HSA - Human serum albumin
IAA - Indole acetic acid
IDP - Inosine 5'-diphosphate
IEF - Isoelectric focusing
kD - Kilodaltons
MES - 2-[N-morpholino]ethanesulfonic acid
mM - Millimolar
MOPS - 3-[N-morpholino]propanesulfonic acid
NADH - β-Nicotinamide adenine dinucleotide, reduced form
NADPH - β-Nicotinamide adenine dinucleotide phosphate, reduced form
NBT - Nitro blue tetrazolium
PAGE - Polyacrylamide gel electrophoresis
PCR - Polymerase chain reaction
PI - Isoelectric point
PMSF - Phenylmethylsulfonyl fluoride
PVDF - Polyvinylidene difluoride
PVPP - Polyvinylpolypyrrolidone
SDS - Sodium dodecyl sulfate
TBS - Tris buffered saline
TTBS - Tris buffered saline containing Tween 20